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EDITORIAL

Dear Reader !

It is our privilege to announce a new and promising development in the life of our Journal: we are going to change its name and from 1994 it will be published as ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA. The change in the main heading covers a basic broadening of our scope. This kind of expansion stands on rather solid ground, as one of the major source of immunology goes back to old age, when microbiologists and physicians started and developed vaccination. Moreover, nowadays infectology represents a complex approach simultaneously involving microbiological and immunological aspects of infectious diseases. We are convinced that large and wide scope of immunology, both basic and clinical may attract many interesting papers and more readers.

Our second aim is to renew the microbiological side, introducing more molecular biology and biotechnology. Our board is ready to start some new columns, like invited reviews, technical leaflets, all-round inquiry on a selected problem, centre-page (a removable figure reviewing one issue). We should like to publish the abstracts of annual National Conferences for Microbiology and Immunology, too.

We have plans to initiate an entirely new series, "Outstanding Papers in Microbiology and Immunology" in which leading scientists will be asked to remember favourite scientific papers affecting their concept fundamentally during their scientific career.

Standing on valuable traditions our board will try to make efforts to refresh the spirituality of ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA. We are intended radically shorten the passing through time for the manuscripts.

The Editorial Board of the Journal accepts regular papers from every topics of basic and clinical microbiology and immunology. Obviously we welcome both experimental and clinical papers in biotechnology and molecular genetics, as well.

Please do not hesitate to ask for further information and honour our Journal with your distinguished attention and personal contribution with your manuscripts.

On behalf of the Editorial Board

Dr. I. Nász
Editor-in-Chief

RECENT DEVELOPMENTS IN RESEARCH OF HIV INFECTION – A “STATE OF ART” (A REVIEW)

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Introduction

It is practically impossible to summarize even the most recent developments of AIDS research, inasmuch as the HIV epidemics are associated with an other epidemic encountered in the AIDS literature. The annual number of publications related to HIV infection increased from 24 in 1982 to 8300 in 1990 [1]. That is why in the following the discussion will be limited to one aspect of HIV infection – the immunopathogenesis. That seems, however, a suitable selection since that is a topic on which our knowledge of HIV infection has been mostly extended and our concepts have mostly changed in the last years.

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HIV, the causative agent of AIDS, principles, failures of and hopes in the antiviral chemotherapy

At the first glance, the first part of the subtitle seems to be a commonplace. It is, however, not the case, since some researchers, the protagonist of which is Peter H. Duesberg, a professor of molecular biology at the University of California, Berkeley, has claimed that HIV is not the etiologic agent of AIDS. Duesberg and his supporters state that HIV is neither necessary nor sufficient for causing AIDS [2]. They either do not present a clear alternative hypothesis or stress that AIDS is induced by pollution or propose that "drug consumption by recently established behavioural groups or conventional clinical deficiencies and their treatments are necessary and sufficient to cause indicator diseases of AIDS" [3].

These views attracted wide publicity in the mass media and can undermine all efforts aiming to control the HIV epidemics. That is why it is most important to contrast them with the findings of the AIDS research which without any exception are in contradiction with these ideas. Let me mention two recent studies which assessed and definitely refuted the arguments of Duesberg and the duesbergians. Schechter and his colleagues [4] followed 715 homosexual men for a median of 8.6 years in Canada; 136 cases of AIDS-related illnesses had been diagnosed in the cohort. Each of them occurred in the 365 HIV-seropositive individuals. In contrast, none of the 350 HIV-seronegative men developed any AIDS-related illness. CD4 counts fell in HIV-seropositive men, but remained stable in HIV antibody-negative men with or without risk behaviour. Similar results were published by Asher et al. [5] in the framework of the San Francisco Multicenter AIDS Cohort Study. During 96 month follow up AIDS developed in 0/215 HIV-seronegative heterosexuals (one of which seroconverted in the study period), in 215/445 homosexual men who were seropositive at the study entry (1984) or seroconverted in the follow up period, and in 0/367 homosexual men who remained HIV-seronegative throughout. No differences were found in the development of AIDS, in the over-all mortality or in the rate of CD4+ cell decline according to use of so-called recreational (sexual arousal stimulating) drugs. These two studies and several others unequivocally support the essential role of HIV in the pathogenesis of AIDS.

Unfortunately, all efforts for finding an anti-HIV compound which as a monotherapy stops replication of the virus in the HIV-infected patients failed so far. The first compound, AZT (Zidovudine, Retrovir) licensed for treatment of AIDS, and the two others, ddI (dideoxy-inosine) and ddC (dideoxy-cytidine) are nucleosides which inhibit an enzyme of HIV, the reverse transcriptase (RT). RT is essential for the formation of a DNA copy from the HIV RNA which later on is integrated in the genome of most cells as a DNA-provirus. Therapeutic trials which were performed in several hundreds of patients indicate that nucleoside RT-inhibitors can prolong the development of AIDS in the late symptomatic stage of HIV disease (see below) and give approximately one extra year to the AIDS patients. Their effect is, however, limited by the development of resistance due to mutations in the pol gene encoding the RT enzyme. Resistance develops even more rapidly, in about one month, against a new class of RT-inhibitors, non-nucleosides. That is why any treatment performed in the

early asymptomatic stage of HIV disease with the existing anti-viral compounds does not seem to be justified. The same conclusion can be drawn from the recent preliminary analysis of the so-called Concorde trial in which early and late AZT treatment protocols were compared [6].

Novel anti-HIV compounds were developed on the basis of detailed study of the virus life cycle in the infected cells. Two types of such drugs, anti-tat and anti-protease compounds were tried in patients recently, the first results being presented at the IXth International Conference on AIDS (Berlin, June 6-11, 1993). Tat inhibitors are analogues of a regulator protein of HIV which was believed to be essential for the replication of the virus. Most recently, however, it was found that tat is non-essential since it can be replaced by any signal (such as TNF α) which induce NF- κ B production in the infected cells (F. Wong Staal, at the Berlin Conference). Accordingly, the first results obtained in patients with a tat-antagonist, Ro 24-7429 were rather disappointing: the drug did not affect CD4+ cell counts or HIV p24 antigen levels during the 12 week treatment period (R. H. Haubrich, at the Berlin Conference). Other compounds, like Ro 31-8959, and A-77003 inhibit HIV *in vitro* through their anti-protease effect. Protease enzyme seems to be essential for HIV for tailoring the final form of some structural proteins. These drugs were found to be more successful in therapeutic trials, decrease in the p24 levels and slight CD4+ cell count increase were observed (several reports at the WS-B26 workshop of the Berlin Conference).

Unfortunately, however, anti-proteases seem to be breakthrough therapeutic agents. Lately, therefore, combination chemotherapy is emphasized. A new type of this form of therapy was proposed and investigated *in vitro* by Chow et al. [7]. The authors combined 3 different RT inhibiting agents: AZT, ddI and a non-nucleoside RT inhibitor, pyridinone. Mutations resulting in the development of resistance against these agents take place at different sites of pol gene encoding the enzyme, that is why the authors hypothesized that an enzyme with so many mutation will lose its functional activity. They called this novel approach convergent combination chemotherapy. *In vitro* experiments supported their expectation: concurrent use of the 3 agents in concentrations equal to, or less than, concentrations measured in the blood of patients on monotherapy with these agents fully prevented the infection with HIV-1 of activated normal peripheral blood mononuclear cell culture and were able to definitely abolish virus replication in the already infected cells. Therapeutic trials with this and similar combination will be started very soon. Most recently, however, Chow et al. revoked their conclusions and admitted that they made a mistake at the evolution of experiment.

Table I
Stages of the HIV disease

Stage	Symptoms	Length	HIV infected cells, %	Level of viraemia	Antibodies		Destruction of the immune system	Activation of the immune system	Viral burden
					anti env	anti core			
1. Acute	50%: no 50%: symptoms of acute viral disease	2-3 months	1:100-1:1000 of CD4+ PBLs	++++	development at the end of stage		unknown	unknown	high
2. Asymptomatic	no	7-12 years in average	1:10000 of CD4+ PBL 1:100-1:1000 in CD4+ cells of lymphoid organs	fluctuation between 0 and +	high titre	high titre	permanent, slow	development at the end of stage	very low
3. ARC (AIDS-related complex)	not life-threatening OI, constitutional symptoms	some months-some years	1:1000-1:10000 in CD4+ PBLs	++	high titre	decreasing titre	permanent, faster than in stage 3	marked, increasing rate	low-moderately high
4. AIDS	life-threatening OI, malignancies	some months-some years	1:100 in CD4+ PBLs	++++	decreasing titres	no or low titres	permanent	marked, increasing rate	high

OI = opportunistic infections

The normal course of HIV infection: changes in time in the distribution, amounts and properties of the virus

Stages of HIV-disease

Typical course of an HIV-infection can be divided into four stages (Table I). Although the longest stage of the disease, the second one is characterized by clinical latency, impairment of the immune system and loss of some immune functions permanently occur even during this stage. That is why it is justified to consider the whole course of HIV-infection as a HIV-disease and an HIV-infected individual as a HIV-patient from the demonstration of his/her infected state which usually coincides with the development of specific antibodies (seroconversion).

The first stage of HIV disease, the acute stage is characterized by a very high level of viraemia. The number of free HIV particles in the plasma and the proportion of HIV-infected CD4+ lymphocytes in this stage is roughly equal to those measured in the AIDS patients [8]. In about 50% of the cases this stage is associated with an acute HIV syndrome the symptoms of which do not differ from the general symptoms of an acute viral infection. The first stage of HIV-disease lasts for about 2–3 months and its end usually coincides with the development of antibodies. Most probably, however, this is only a coincidence [9], antibodies and even neutralizing ones have no decisive role in the termination of the first stage, which is characterized by a sudden dramatic decrease in the number of free virus particles and the rate of HIV-infected cells. At the beginning of the second stage only a minor proportion, about 1 out of 10 000 CD4+ cells is infected with HIV, and the level of viraemia is also extremely low, although some fluctuation can be observed over time [8].

In the second stage of HIV disease, the vast majority of virus burden can be found in the lymphoid organs, in the lymph nodes, adenoids and tonsils [10, 11], as well as in Peyer-plaques (A. S. Fauci, at the Berlin Conference). According to these recent publications, HIV is active in the lymphoid tissue throughout the period of clinical latency. An extraordinarily large number of CD4+ lymphocytes and macrophages was found to be infected with HIV in the lymphoid organs. In the vast majority of these cells, however, HIV-infection was found to be latent, non-productive. Most RNA in the lymph nodes was associated with cell-free HIV particles. These virions were found to be present as virus-antibody or virus-antibody-complement complexes on the surface of the follicular dendritic cells (FDC) of germinal centres of lymph nodes. Clearly, lymphoid tissue can be considered as the major reservoir for HIV in the second, asymptomatic stage of HIV disease and may have a role in the transmission of the virus to new cells. It is tempting to speculate, however, that trapping of HIV on the FDCs can be an important protective mechanism against the progression of HIV disease. The major argument for this assumption is as follows: in the third and fourth, symptomatic stages of HIV infection, degeneration and death of

FDCs can be observed, ultrastructure of lymph nodes is destroyed and virus trapping is virtually absent [10].

The third stage of HIV disease (frequently called as AIDS-related complex, or ARC) is associated with an increase in the rate of HIV-infected blood lymphocytes and tissue macrophages, and a higher level of viraemia. Increase of the viral burden is associated with the first, not life-threatening infections and constitutional symptoms.

The last stage of HIV-infection is the full-blown AIDS with a further increased HIV-replication and the development of life-threatening opportunistic infections and malignancies.

Possible cause of permanent decrease of the CD4+ cell count in the blood of HIV-infected patients

The hallmark of progression of HIV disease detailed above is the permanent fall in the percentage and absolute number of CD4+ lymphocytes. AIDS develops when the CD4+ cell count reaches a critical value. This value is usually considered to be 200/ μ l, and according to the recent CDC definition introduced in the USA but not accepted for Europe, each HIV-infected patient with a CD4+ cell count of less than 200/ μ l can be considered as AIDS patient regardless of his or her clinical state.

Table II

Potential mechanisms of the depletion of CD4+ lymphocytes in HIV disease

1. Direct HIV-mediated effects

- cytopathic effects: single cell killing
- cytopathic effects: HIV-mediated formation and fast destruction of syncytia from HIV-infected and not infected CD4+ cells

2. Virus-specific immune responses

- HIV-specific cytolytic lymphocytes
- antibody-dependent cellular cytotoxicity
- antibody and complement mediated killing of infected and not infected lymphocytes
- autoimmune mechanisms caused by cross-reaction between some HIV and host cell antigen structures

3. Anergy and apoptosis of uninfected CD4+ cells due to

- inappropriate cell signalling through gp120 or gp120-anti-gp120 immune complexes
- HIV-related and other superantigens
- defective antigen presenting cell functions

4. Defective maturation of CD4+ cells in bone marrow and thymus

One of the most controversial issue of AIDS research is the study of the causes of the CD4+ decrease. Many theories were put forward, the most important of which are summarized in Table II.

The depletion of the CD4+ cells can be due to the cytopathogenic effect of HIV or to the quick destruction of the syncytia which form at least under some circumstances in vitro from HIV-infected and not infected cells. Besides of these processes directly related to the virus replication, different types of other, indirect phenomena, like virus-specific immune responses, autoimmune mechanism or programmed cell death can be also responsible for the permanent decrease of the CD4+ cells. Recent experiments performed in SCID mice seem to support the role of indirect mechanisms (D. E. Mosier, at the Berlin Conference). SCID mice are tolerant for cells of different species, therefore they can be reconstituted with lymphoid tissue of human origin. The "humanized" animals can be infected by HIV. Mosier and his co-workers compared the rate of CD4 depletion in such animals infected with different HIV-1 strains. Paradoxically, infection with strains which exhibited a strong cytopathogenic effect in vitro resulted in little or no depletion of CD4 cells, whereas in vitro non-cytopathogenic strains induced a marked CD4+ cell depletion.

Several other observations also support the role of indirect mechanism in the CD4+ cell decrease. For example Daniel et al. [12] observed a significant proportion of the CD4+ cells in peripheral blood of HIV patients to be covered with HIV-anti-HIV immune complexes and found a strong direct correlation between the percentage of these cells and the progression of HIV disease.

The possible role of the programmed cell death (apoptosis) was raised first by Luc Montagnier and his co-workers. They found that T cells from HIV-infected individuals die markedly more quickly in culture, than cells of HIV-negative individuals and dying occurs mainly by apoptosis. Several processes were implicated in the development of T cell apoptosis, like the hyperactivation induced by the HIV envelope glycoprotein gp120 [13], or gp120-anti-gp120 immune complexes [14]. The role of defective antigen presenting cell function in the development of apoptosis of CD4+ cells was also supposed [15]. The eventual role of apoptosis-induced cell death in the in vivo depletion of CD4+ cells is supported by observations obtained in chimpanzees. These animals can be infected by HIV-1 but they do not develop HIV-related disease and no evidence for apoptosis of their CD4+ cells were found [15].

Apoptosis, however, affects both CD4+ and CD8+ T cells, and so cannot be the primary mechanism of CD4+ lymphocyte depletion. That is why besides an increased rate of the destruction of the CD4+ cells a defective maturation process also should be seriously considered. Both bone marrow stem cells and thymic epithelial cells were found to be infected by HIV, which may lead to a defective lymphocyte maturation. The selectivity for CD4+ cells, however, cannot be easily explained by this assumption. That is why, most recently Helbert et al. [16] developed a hypothesis according to which the defective antigen presentation is the key factor in CD4+ depletion. Because of impaired antigen presentation by HIV-infected dendritic cells, CD4+ T cell population decrease in number, due not to their destruction but because they fail to expand in response to antigenic stimulation. Initially impairment develops in CD4+ lymphocytes expressing CD45-RO antigen (memory or hyperreactive cells). In later stage of HIV infection, antigen presentation is further impaired as macrophages also become infected and deficient which leads to shrinking of the CD45 RA+ (naive or quiescent) CD4+ pool as well.

Immune activation and immunosuppression in HIV patients. The case of TH1-TH2 switch

Although CD4+ depletion is considered to be the hallmark of the progression of HIV disease, it is well known for a long time, that some defects of immune function can be detected very early when the CD4+ cell count is still normal. Now it is more and more clear that these defects have a major role in the immunopathogenesis of HIV infection as well. Interestingly enough, however, this immunosuppression is strongly associated with the activation of the immune system and both processes contribute to the progression of HIV disease.

Activation of the immune system in HIV infection, increase in the level of some inflammatory cytokines

HIV infection is associated with some changes in the level of inflammatory cytokines. In the late, symptomatic stages HIV disease, markedly increased TNF-alpha, IL-1, and IL-6 levels can be observed. The high levels of these cytokines, first of all those of TNF-alpha can be directly responsible for the development of some symptoms of HIV disease, such as weight loss, fever, etc. On the other hand, elevated TNF-alpha levels can result in increased production of HIV particles in the infected cells, through an increased transcription of HIV mRNA due to NF- κ B stimulation. That is why question of Matsuyama et al. [17] "...Is AIDS a TNF-alpha disease?" seems to be right.

If immune activation has a major role in the progression of HIV disease, its inhibition can be used for therapeutic purposes. Most recent data indicate that it is really the case. Schwarz et al. [18] summarized the clinical course of 53 HIV-infected kidney-grafted patients, and found that the 5-year cumulative incidence of AIDS was significantly ($p = 0.001$) lower in 40 transplant patients with an immunosuppressive regimen including cyclosporin A (31%) than in the 13 transplant patients receiving other immunosuppressive regimen (90%). Similarly, Pantaleo et al. [19] found cyclosporin A to suppress HIV infection in primary T lymphocytes acutely infected in vitro at concentrations that corresponds to easily tolerated serum levels of drug during the treatment of other diseases. Fauci at the Berlin Conference reported on the ability of an IL-1 inhibitor and an anti-TNF-alpha monoclonal antibody to effectively suppress HIV-infection in vitro.

Immunosuppression independent of CD4+ cell loss in HIV infection

Pioneer studies of Clerici and Shearer (reviewed in reference [20]) which were supported by the findings of other groups [15] showed that there is a sequential and progressive loss of TH1 function such that the TH1 response to recall antigens is the first to be lost, followed by loss of TH1 function to alloantigens (major histocompatibility complex), and finally by loss of reactivity to PHA. This conclusion

is based on the analysis of more than 600 HIV+ asymptomatic individuals. When cells of these individuals were stimulated by recall antigens or mitogens, it has been observed that progression to AIDS is characterized by loss of IL-2 and IFN-gamma production, concomitant with increases in IL-4 and later on IL-10 production. Since the first two cytokines are produced by the so-called TH1 subclass of T helper cells, whilst IL-4 and IL-10 are produced by the so-called TH2 cells, these findings suggest that progression to AIDS is dependent to a TH1-TH2 switch. TH1 cells are responsible mainly for cellular immunity, whereas humoral immunity and first of all allergy is associated with the TH2 cells. A relative dominance of TH2-mediated responses, such as B-cell hyperactivation or elevated serum IgE levels can be recognized during HIV infection (reviewed in reference [21]). The reasons for the TH1-TH2 switch are not known yet, but defective destruction of TH1 cells due to defective antigen presentation can contribute to this process.

These observations suggest that there is an IL-2 deficiency in HIV-infection, and IL-2 substitution can be a reasonable therapeutic approach. Early attempts to use IL-2 for therapeutic purposes failed, since IL-2 treated patients progressed even quicker to AIDS than the untreated patients. Now Kovacs et al. [22] using a combined treatment of IL-2 and antivirals (AZT and/or ddI) obtained markedly better results in 8 patients with CD4 counts above 200/ μ l. Eighteen million international units of IL-2 were administered to the patients for 5 days every 2 months. Four out of 8 patients had an average 100% increase in CD4+ cell numbers by 6–10 months, in 2 patients an improved blastogenic response to tetanus toxoid was observed, too. These findings suggest that IL-2 may be an effective adjuvant to antiviral treatment in at least a part of HIV-infected patients.

Role of humoral factors in the progression of HIV disease

More and more data have been accumulated recently indicating that the humoral factors (specific antibodies, complement) which in most infectious diseases have major role in the defence against, and in the elimination of the causative agents may have a harmful effect in HIV infection, facilitate the progression of HIV disease. Antibodies with this harmful effect are called enhancing antibodies. There are two types of these antibodies, complement-dependent and complement-independent ones.

These antibodies were detected first in vitro [23, 24]. Recent findings of Homsy et al. [25] and our group [26], however, suggest that both complement-independent and complement-dependent enhancing antibodies may have clinical relevance. Our newest results (Fig. 1A) indicate that appearance of complement dependent-enhancing antibodies predicts the development of AIDS in asymptomatic HIV-infected patients [27].

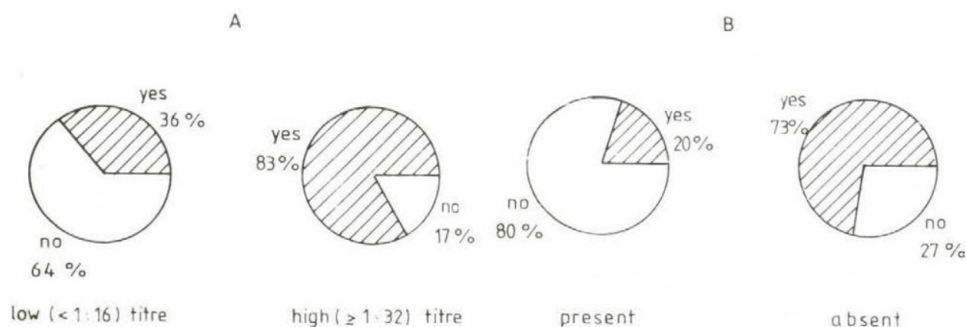


Fig. 1. Development (yes) or no development (no) of AIDS in patients with low and high titre of complement-dependent enhancing antibodies (A) and in patients in which complement-dependent neutralizing antibodies are present or absent (B) in a 55–64 month follow-up period

An other type of antibodies, IgG and IgA type autoantibodies against the Fab part of IgG, seem to have major role in the progression of HIV disease as well. According to the studies of Süsal et al. [28] there is a highly significant negative correlation between the titres of these antibodies and the CD4+ cell count in longitudinally tested HIV patients.

Complement itself without any antibodies can contribute to immunopathogenesis of HIV infection (reviewed in [29]). HIV particles were found to activate the classical pathway of complement, whereas HIV-infected cells activate complement through both the classical and alternative pathways. Fixation of complement fragments, such as C1q or C3b can contribute to infection of cells carrying complement receptors (activated T cells, monocytes, macrophages) and can also facilitate trapping of HIV particles on the surface of FDCs in the lymph nodes.

Is there a protective immunity against HIV?

Till the latest time, the answer to this question was: probably not. Only the recently published studies of Clerici and Shearer [20] demonstrated that protective immunity against HIV may exist. These authors studied IL-2 responses to HIV envelope peptides of lymphocytes of more than 100 HIV seronegative and PCR-negative individuals at risk for infection with HIV. These individuals were either permanent sexual partners of HIV seropositive patients, or intravenous drug abusers sharing needles and syringes with HIV infected persons, health care workers accidentally injured with HIV-contaminated needles or new-born infants of HIV-infected mothers. Members of these groups in 39–72% responded to env peptides by IL-2 production. In contrast, only 7/167 (5%) of presumed unexposed adult HIV-seronegative individuals and 0/11 of new-borns of HIV-seronegative mothers responded to one or more peptides.

These findings raise the possibility that a marked proportion of HIV-exposed individuals – although they have encountered HIV and become primed to the virus – persist in TH1-reactive, seronegative and most probably virus-free state. These findings support the possibility of a cell-mediated immune protection. According to Clerici and Shearer [20] it may result in an ability to protect against a higher intracellular load. Once, however, the infection outrun the cell-mediated immune response, HIV-infection will be permanent and antibodies develop.

Protective mechanisms against the progression of HIV disease and the causes of their failure

Several evidences support the existence of such protective mechanisms. First of all, there is a formal logical argument: if there were no protective processes against the progression of HIV infection, HIV disease would be an acute fatal illness which would kill most infected persons in some months. There are some, fortunately very rare examples for such a rapid progression of HIV disease. The best evidence for an effective protection are, however, the existence of so-called long-term-survivors.

Long-term-survivors of HIV-disease

Unfortunately, the term long-term-survivor (LTS) is a rather misleading and equivocal one. In the following, I shall use this terminology for HIV-infected persons with no or only slight impairment in their system even after 8–10 years after HIV-infection. It seems that roughly 10% the HIV-infected persons belong to this group. In Fig. 2 the results of a 5-year longitudinal measurement of CD4+ cell counts in two Hungarian haemophiliacs are shown in order to illustrate LTS state. These two patients (two cousins) were infected by imported factor IX concentrate in 1982. Ten years after their infection, their CD4 cell count and percentage is perfectly normal, they are asymptomatic, and there is not a sign of immune activation (increased percentage of DR+ lymphocytes, elevated neopterin or immune globulin levels). At the Berlin Conference an international task force was formed in order to initiate a complex study of the LTSs.

Nature of the protective mechanism

The study of LTSs would be most important since our knowledge about the nature of protective mechanisms which hinder the progression of HIV disease to AIDS is incomplete. Immune mechanisms that protect against other viral diseases probably contribute to this protection. There are neutralizing antibodies in the blood of patients in the early stage of HIV-disease but they are missing from the blood of late phase patients. Loss of neutralizing antibodies can predict progression to AIDS according to our recent studies [27] (Fig. 1B). Plasma from individuals in the early stage of HIV

disease can be used for a passive immunization of patients with late HIV disease. According to Karpas and Bainbridge [30] this form of treatment induced a long-lasting (6–32 month) clinical remission in AIDS patients.

Cell-mediated specific immunity (CTL) also may have an important role in the protection against disease progression. The efficiency of CTL is also decreasing in the late stage of HIV-disease but some activity is present even in the AIDS patients.

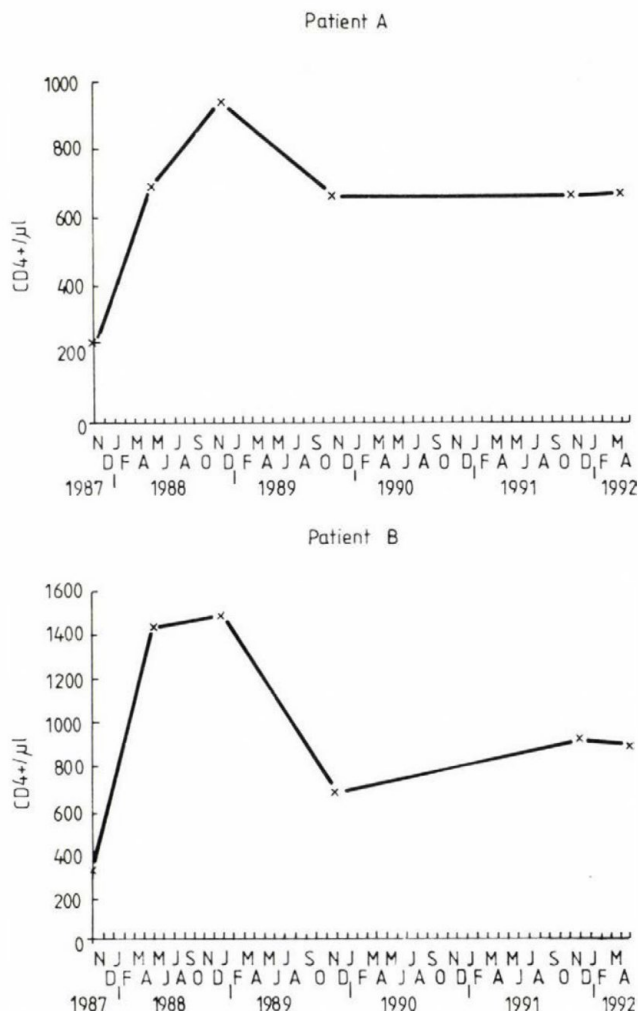


Fig. 2. Changes in the absolute number of CD4+ lymphocytes in two long-term survivor HIV-infected patients with haemophilia B. The patients are cousins and were infected by imported factor IX concentrates in 1982 or 1983

Jay Levy and his group were the first to describe a process that may be a decisive protective mechanism and which was found to be lost in late phases of HIV disease but remains at high level in LTSs [31]. This CD8+ cell-dependent mechanism consists of a non-MHC directed non-cytopathogenic suppression of HIV replication mediated by a novel low molecular weight cytokine (different from any known cytokines). This HIV-suppression is associated with an activated CD8+ cell with a cytotoxic CD28+ phenotype.

Possible causes of the failure of protective mechanisms

In the vast majority of HIV-infected individuals the protective mechanisms fail over time and disease progresses to AIDS. Possible causes for this failure is summarized in Table III. Changes in both the virus and the host are responsible for the failure. As it was mentioned before, the viral burden is markedly higher in the body of symptomatic HIV patients than in those in the early stage of disease. Moreover, strains with low virulence, replicating slowly and in low amounts (slow/low strains), unable to induce syncytia (NSI strains), give place to more virulent strains growing in vitro rapidly and in a high amount (rapid/high strains) which readily induce syncytia in MT-4 cells (SI strains).

Table III

Potential causes of failure of protective mechanism against the progression of HIV disease

1. Changes in the virus

- switch from dominance of strains with low virulence (slow/low, NSI*) to strains with high virulence (rapid/high, SI**)
- increasing replication rate leading to increasing mutation rate
- increasing viral burden

2. Changes in the host

- progressive activation of the immune system
- TH1-TH2 switch
- switch from dominance of neutralizing antibodies to that of enhancing antibodies
- exhaustion of the existing protective mechanisms, first of all marked decrease in the activity of the HIV replication inhibiting CD8+ cells

*NSI, non syncytium inducing

**SI, syncytium inducing

There is no agreement about the sequence of events leading to disease progression. Some experts believe that the change in properties of HIV strains in the body of infected persons is the primary event and the increase of viral burden results in the exhaustion of protective mechanisms.

Jay Levy in Berlin [31] raised a hypothesis in which he tried to work out a unified concept. Figure 3 which was drawn according to this lecture, shows the hypothesis. According to Levy, the primary event is the TH1-TH2 switch which was detailed above. In the consequence of this switch production of IL-2 diminishes whilst that of IL-10 markedly increases. In the other hand, IL-2 markedly facilitates, whereas IL-10 strongly inhibits the HIV-replication blocking capacity of CD8+ cells. That is why, dominance of the TH2 cells leads to a dramatic decrease of this activity of CD8+ cells which in turn results in a markedly increased replication of the virus. Increase in the replication rate leads to increased mutation rate of HIV and emergence of more virulent strains. This assumption – if it can be proved – indicate that the reversal of the TH1-TH2 switch can hindered or even turn back progression of HIV disease. According to the most recent results, there is one cytokine, IL-12 which is able to do this reversal [32]. Since the level of IL-12 is markedly decreased in the late stage HIV disease [33], IL-12 treatment can be a promising new therapeutic strategy in the future.

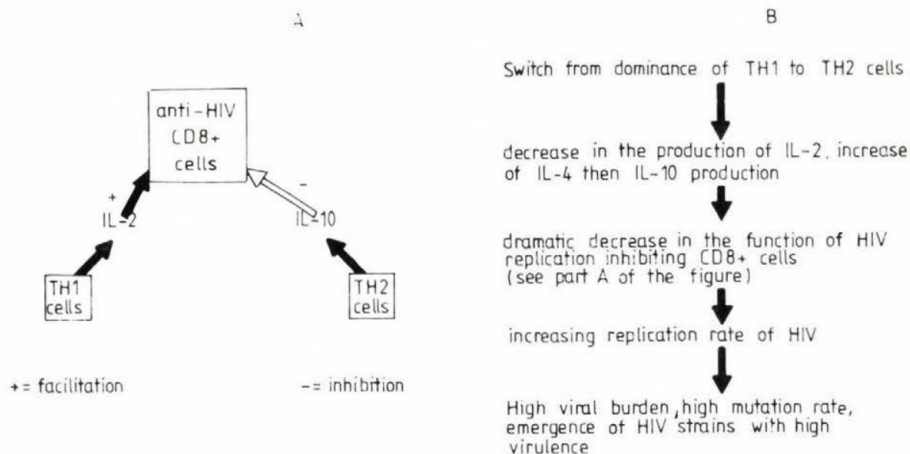


Fig. 3. Possible causes of the failure of protective mechanisms against the progression of HIV disease (according to J.A. Levy [31]), for details see the text

Do not let the best to be the enemy of the good: the HIV vaccines

The slogan of the Nobel-prize winner biochemist, Temin was cited by Dani Bolognesi [33] who gave an overview on the preventive HIV vaccines. The slogan can be considered either justified or very dangerous in the case of HIV vaccination. Let me outline two scenarios according to these two possibilities.

The worst scenario: the HIV vaccination will not prevent the infection of the HIV exposed people or it even facilitate their infection. The most dangerous consequence of vaccination would be the development of enhancing antibodies. Very few studies addressed this possibilities, no enhancing antibodies were detected, however, so far in human volunteers vaccinated with different HIV candidate vaccines. Efficacy of HIV vaccines was seriously doubted by the unexpectedly bad results of SIV vaccination which used to be considered as a model of HIV vaccination. According to the newest findings [34] all protection by inactivated SIV vaccines in over 200 monkeys was due to the presence of human T cell antigens in the vaccines and in the challenge viruses and not to SIV components. Attempts to protect macaques against SIV by using purified recombinant proteins from the envelope or core of SIV have been unsuccessful although in some vaccinated animals strong SIV-specific neutralizing antibody and cellular immune responses were found. A third argument against the current practice of experimental HIV vaccination was raised by Jonas Salk last year at the VIIIth International Conference on AIDS. On the basis of the results of Shearer' group on the unique role of cellular immunity in the protection against HIV (see above), he claims that exclusively vaccines that raise only cellular immunity and do not result in antibody development can be efficient in preventive vaccination.

The best scenario: HIV vaccination will render the vaccinated people resistant for HIV infection. The vaccinated individuals will develop mucosal immunity which blocks the entry of the virus in the body of the sexual partners through rectal or vagina mucosa and if nevertheless some host cells are infected, they will be destroyed by an effective cellular immune response. The most encouraging results emerged from chimp experiments. Several types of HIV-1 vaccines, including subunit gp120 and gp160 vaccines, as well as combined regimens of whole inactivated vaccines and peptides were found to protect chimpanzees against free HIV and HIV-infected homologous lymphocytes.

In the newest, phase I-II therapeutic trials in human volunteers a relatively stable humoral immune response was obtained and at least some vaccines raised cellular immune response as well. The levels of these immune parameters was equal, or close, to those measured in chimpanzees when they were found to be protected against infection with the wild type of virus.

It seems most probable that phase III, large-scale efficacy trials of candidate vaccines will be started in several thousand HIV-seronegative individuals soon, in 1994-1995, in some countries with high HIV prevalence and incidence. According to model calculations in such countries even vaccines with a 50% efficacy can slow down or control the HIV epidemics.

Besides of prevention, HIV candidate vaccines can be used for immunotherapy as well. Several groups are studying this possibility. The initial results were promising, a large-scale study with 3 different vaccines in almost 10 000 HIV infected patients will be performed in the USA in the near future. The reason for administrating HIV vaccines for already infected persons are as follows: (a) vaccines can raise protective humoral and cellular immune processes which do not develop during natural infection and (b) vaccines can inhibit processes, like TH1-TH2 switch which lead to the exhaustion of protective mechanisms against progression of HIV disease.

Concluding remarks

Recently published findings indicate that the interest of AIDS researchers is slowly moving from study of life cycle of HIV in different target cells towards investigation of the fate of HIV in the whole body of the infected patients. It seems reasonable to hope that better understanding of immunopathomechanisms of HIV disease will result in new therapeutic approaches which – in combination with antiviral drugs – may maintain patients with HIV disease in asymptomatic state.

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SOME CHARACTERISTICS AND PARTIAL PURIFICATION OF THE *GANODERMA LUCIDUM* CELLULASE SYSTEM

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The extracellular cellulase system of the white-rotting basidiomycete *Ganoderma lucidum* was characterised while growing in cellulose-containing shaken liquid culture. The protein content of the culture filtrate reached its maximum after 36 days and cellulase activity at about 60 days. Different cellulase activities (endoglucanase, cellobiohydrolase and β -glucosidase) were determined in a range of pH extending from 6 to 2. All of the three enzyme activities have at least three peaks between pH 6 and 2, although optimum points of the different enzymes are slightly different, showing that the enzyme complex consists of a number of enzymes and isozymes. Partial purification of the enzyme complex was carried out by DEAE-cellulose column chromatography. Using 0–3 M linear urea gradient, protein was eluted in one sharp peak corresponding mainly to β -glucosidase activity. Comparing crude extracellular protein with that of purified by the column using PAGE indicated that this method was suitable for the separation and partial purification of one type of *Ganoderma* cellulases.

Cellulases, the groups of enzymes capable of hydrolysing insoluble cellulose to glucose are mainly produced by bacteria and fungi. Cellulose may be hydrolysed by the combined action of at least three enzymes: cellobiohydrolases (exo-1,4- β -glucanases, EC 3.2.1.91), endoglucanases (endo-1,4- β -glucanases, EC 3.2.1.4) and cellobiases (β -glucosidases, EC 3.2.1.21). In the last two decades a great progress has been made in the description of all the principal enzymes of cellulase system. Especially molecular characteristics (MW, AA-composition, absorbability to cellulose, catalytic activity, etc.) of cellulases in industrially important fungi have been studied exhaustively [1, 2]. Noteworthy is, however, that these results are mostly confined to the enzymes of a small group of moulds such as *Trichoderma*, *Penicillium* and *Aspergillus* species [3–6] and to the best-known lignocellulose-degrading

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basidiomycete, *Phanerochaete chrysosporium* [7]. The data concerning the cellulolytic enzymes of wood-rotting basidiomycetes are scarce, though they seem to differ remarkably from that of both moulds and bacteria. *Ganoderma lucidum* (Poriales, *Ganodermataceae*), a white-rot fungus of high cellulolytic and ligninolytic ability [8] has also an importance as producing terpenoids, polysaccharides and alkaloids of much benefit to medicine [9]. A number of *Ganoderma* enzymes were investigated [10–13], but cellulases have not been studied yet since they strongly absorb on their substrates. A DEAE-cellulose chromatography method has been developed to separate one type of cellulases from *Ganoderma* culture filtrate. Catalytic activity, pH optimum and electrophoretic pattern of the partially purified enzyme were examined.

Materials and methods

Cultivation. *G. lucidum* was obtained from the mycelium collection of the National History Museum, Budapest. The fungus was grown on malt extract agar and subcultured every two months. For cellulase production 350 ml of modified Czapek solution (containing 0.5 g/l glucose and supplemented with 1 g/l yeast extract and 10 g/l filter paper as cellulose source) was inoculated with 3 mycelium discs of 0.5 cm diameter cut from malt agar plates and cultivated by shaking in 1 l Erlenmeyer flasks for 50 days. Though glucose inhibits the synthesis of cellulases, a certain concentration was necessary to indicate mycelial growth.

Enzyme assays. Cellobiohydrolase activity was assayed using filter paper (FPase activity) and endoglucanase using CMC (Na-carboxymethyl-cellulose, CMCase activity) as substrates, while β -glucosidase was determined by the PNPG (paranitrophenol- β ,D glucoside) method, as described by Ghose et al. [14].

Protein content determination. Soluble protein content of culture filtrate and DEAE-column fractions were determined according to the method described by Bradford et al. [15]. Changes of protein content during column chromatography were monitored by measuring the absorbance at 280 nm.

Adsorption to DEAE-cellulose. For determination of cellulases to DEAE-cellulose a batch adsorption was used. One gram aliquots of activated and dried cellulose were stirred for an hour at 4°C with 4 ml of culture filtrate of *G. lucidum*. After decantation of supernatants (non-adsorbing protein fraction) the DEAE-cellulose aliquots were washed with 4 ml of different eluents, namely: 1 M KCl; 1.5 M KCl; 1 M urea; 2 M urea; 3 M urea, 5 M urea, 1 M KCl combined with 1 M urea and 1.5 M KCl combined with 1 M urea. Adsorption of cellulases were determined by measuring the protein content of supernatant and eluents after the adsorption and elution periods.

DEAE-cellulose column chromatography. DEAE-cellulose was equilibrated with glucose-free Czapek solution. For batch adsorption 50 ml of DEAE-cellulose stirred in Czapek solution was incubated with 350 ml culture filtrate of *G. lucidum* at 4°C for 1 h. After the adsorption at 10 mm $\times$ 70 mm column was prepared. Having collected the culture filtrate containing the non-adsorbed protein fraction, the column was washed with a urea gradient ranging from 0 to 3 M dissolved in modified Czapek solution. Fraction volume was 3 ml. Cellobiohydrolase, endoglucanase and β -glucosidase activities, A_{280} absorbances and protein content were determined from each fraction.

Polyacrylamide gel electrophoresis (PAGE). Protein content of 110–115 samples of DEAE-column was precipitated by 2.5 volumes of 96% cold ethanol and redissolved in 0.05 M citrate buffer pH 5.0 giving a final concentration of 250 μ g/ml protein; 20 μ l of the samples were loaded on 8% native slab gel. The electrophoresis was carried out at 4°C, using Tris-glycine buffer pH 8.3 and 15 mA/slab. Samples of precipitated culture filtrate were prepared and applied for PAGE by the same way. Gels were stained for protein by Coomassie Brilliant Blue (CBB) [16].

Results

Protein content and cellulase activity of *G. lucidum* cultures were determined from samples taken in several different times from 60-day-old shaken cultures. The changes of the cellulase activity can be described by β -glucosidase. The results are shown in Fig. 1. Beta-glucosidase activity appears only at about the 30th day. This is partly caused by the inhibitory effect of glucose given as starter. Protein content increases slowly during culture period and slightly decreases at the end of it, which can be explained by the effect of proteases released. Beta-glucosidase activity increases suddenly at the end of the culture period. This may be explained by the fact that a number of enzymes (mainly some type of β -glucosidases known to be bound to the cell wall) are released in large quantities only at the late period of cultivation, as it has been proved several times [17, 18].

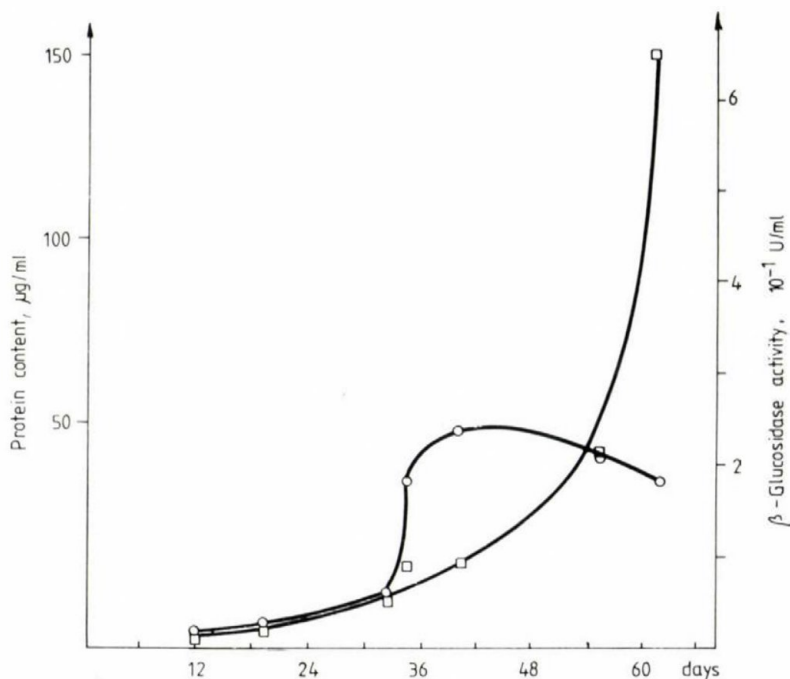


Fig. 1. Changes of protein content (○) and β -glucosidase activity (□) in liquid culture of *G. lucidum* during cultivation

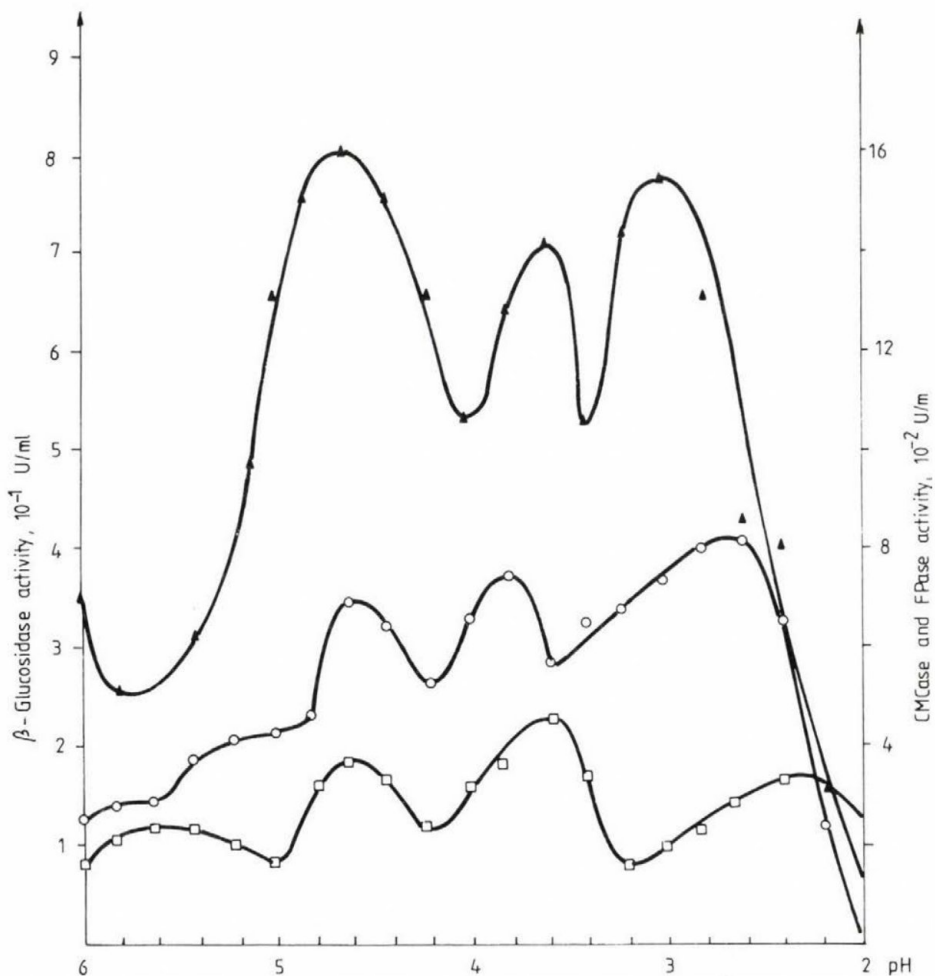


Fig. 2. Changes of cellulase enzyme activities plotted against pH in samples of 60-day-old liquid culture of *G. lucidum*. ▲ β -glucosidase, ○ CMCase, □ FPase activity

The different cellulase activities determined from the medium at the end of cultivation are highly dependent on pH used in the tests. It is known that in contradiction to bacterial ones, fungal cellulases have their optimum pH in acidic ranges [1]. Accordingly, activities were determined in a range of pH extending from 6 to 2. The results of this investigation are seen in Fig. 2.

Among cellulase activities β -glucosidase turned out to be the highest (about 8×10^{-2} U/ml). CMCase and FPase activities reached 8×10^{-1} U/ml and 4×10^{-2} U/ml, respectively. Depending on pH change all of the three enzyme activities (FPase, CMCase and β -glucosidase) have at least three peaks between pH 6 and 2, although the optimum points of the different enzymes are somewhat different. This shows clearly that the enzyme complex of *Ganoderma* consists of a number of enzymes and isozymes.

Column chromatography is widely used for separation of cellulases [19]. Boyer et al. [20] combined ion exchange chromatography with gelfiltration using substituted celluloses for the separation of fungal cellulases. According to our previous findings DEAE-cellulose is suitable for adsorbing and concentrating cellulases from liquid media. In the case of *Aspergillus* and other mould cellulases such columns can be easily eluted with 0.1–0.2 M KCl gradient. Cellulases of many basidiomycetes (especially wood rotting fungi) however, act very differently from the above types as elution rate decreases with increasing ionic strength. According to our results of batch experiments, 0–3 M/l urea was suitable for partial elution of *Ganoderma* cellulase adsorbed by DEAE-cellulose. Adding KCl to urea, adsorption increased and elution was weak (Table I).

Table I

Batch experiment with the *G. lucidum* culture filtrate using different combinations of KCl and urea for elution.

The adsorption rate of enzymes (protein content of the eluent) is described by A_{280} .

Sample	A_{280}
Culture filtrate before adsorption	1.950
Culture filtrate after adsorption	0.735
Elution by 1 M KCl	0.212
Elution by 1.5 M KCl	0.056
Elution by 1 M urea	0.982
Elution by 2 M urea	2.318
Elution by 3 M urea	2.450
Elution by 5 M urea	2.458
Elution by 1 M KCl combined with 1 M urea	0.209
Elution by 1.5 M KCl combined with 3 M urea	0.479
Elution by Czapek solution	1.052

Figure 3 shows the chromatographic profile of a DEAE-cellulose column, eluted with 0-3 M linear urea gradient. Elution resulted one sharp peak at 2.8 M urea. The fractions 110-115 corresponding mainly to β -glucosidase activity were pooled, precipitated with ethanol and the protein was compared with that of the crude culture filtrate using PAGE. Densitogram of the gel is seen in Fig. 4. Crude extracellular *Ganoderma* protein (A) consists of 9 bands, while the purified one corresponding to the column's peak (B) shows only 7 bands differing in R_f or intensity from those of the crude protein. The main band of A (peak 6) and peak 4 are absent in B, so these are perhaps not cellulases. Peak 7 is much higher in B, than in A. Probably this peak contains β -glucosidase of *Ganoderma* (molecular weight about 27 000). This experiment shows that DEAE-cellulose chromatography has resulted in a significant purification and enrichment of *Ganoderma* cellulase.

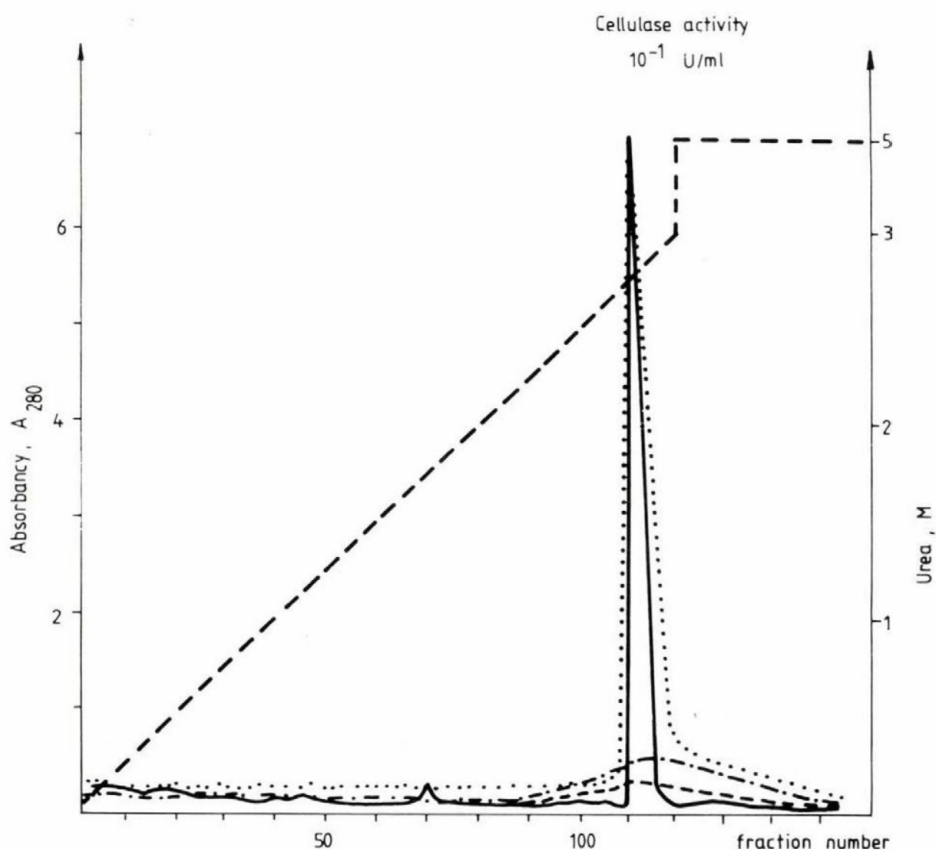


Fig. 3. Profile of DEAE-cellulose column chromatography of *G. lucidum* cellulase.

..... β -glucosidase, - · - · - · - FPase, - - - - CMCase, — A_{280}

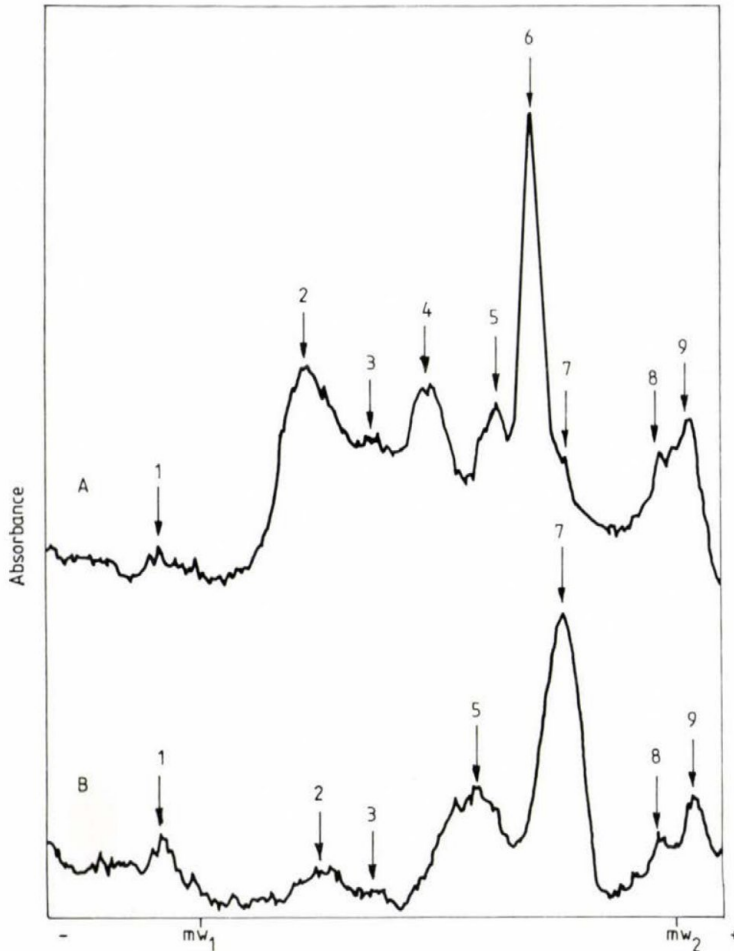


Fig. 4. PAGE densitogram of *G. lucidum* extracellular protein. A: crude protein of culture filtrate, B: cellulase purified by DEAE column chromatography, mw_1 : molecular weight standard 67 000, mw_2 : molecular weight standard 14 400

Discussion

The growth and the cellulase production of *G. lucidum* in shaken cultures is rather slow. Compared to a 4-to-7-day culture period of cellulase producing imperfect fungi used commonly in practice, it takes a long time that cellulases appear in the culture filtrate of *Ganoderma*. The quantity of cellulases produced in liquid culture is

also much less than produced on solid media. Especially the endoglucanase and cellobiohydrolase production is much lower than in the solid phase [8].

As known from the literature, the individual activities of cellulase complexes are generated by several isozymes, differing only slightly in structure and pH optimum. That is correct in the case of *Ganoderma*, too. Both β -glucosidase and CMCase or FPase activities have at least three well-defined peaks between pH 6 and 2. Activities are uncommonly high in the acidic range up to pH 2. Optimum points of pH of the individual activities are somewhat different, and this causes an almost equal degradation ability in the whole range whilst pH changes into acidic direction during cultivation. The increase of acidity is common in cellulolytic cultures of fungi, especially *Poriales*. This is caused by secretion of oxalic acid, citric acid and other Krebs-cycle intermediates. This phenomenon plays a double role in cellulose degradation: beyond assuring the optimum acidic pH for enzymes, it gives a great selective advantage for fungi in the medium as almost no other organisms can tolerate acidic conditions like that.

Our further experiments done with *Aspergillus flavus*, *Phellinus pomaceus* and *G. lucidum* cellulases have confirmed that adsorptive characteristics of cellulases of *Ganoderma* and other wood-rotting fungi are quite different from that of the widely used cellulolytic fungi (e.g. *Aspergillus*). The adsorption of *Ganoderma* cellulase to its substrate is very strong and increases in direct ratio with ionic strength, causing changes in conformation of the molecule. This is in accordance with the model that highly absorptive "full-value" cellulase complexes exist, in which cellulase activity is in direct ratio with adsorption rate [1]. Presumably this is why *Ganoderma* and other *Poriales* possessing highly adsorptive enzymes can degrade lignocelluloses faster in solid than in liquid phase.

DEAE-cellulose column chromatography was available for the separation and partial purification of one type of *Ganoderma* cellulases using 0–3 M urea gradient. Although peak fractions possessed mainly β -glucosidase activity, they did not contain a single peptide, but exhibited 7 bands in PAGE. This may lead to the conclusion that β -glucosidase of *G. lucidum* should be consisting of different isozymes as suggested by the results of pH range experiments as well. Further investigations and comparison made between this enzyme complex and that of other wood-rotting fungus species would be important for clarifying the special properties of strongly bound basidiomycete cellulase systems.

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COMPETITION BETWEEN DOPAMINE, BETA-RECEPTOR AGONISTS AND PROMETHAZINE IN *ESCHERICHIA COLI* PLASMID REPLICATION

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The substrates in the phenylalanine, metabolism play key roles in the physiological processes of bacteria. Promethazine affects the phenylalanine metabolism in *Escherichia coli*. The antibacterial and antiplasmid actions of promethazine were prevented by phenylalanine, tyrosine, phenylpyruvic acid, phenylacetic acid, noradrenaline and dopamine in minimal medium. Isoproterenol (and phenoxybenzamine) reduced, while propranolol, oxyprenolol and alprenolol isomers enhanced the antiplasmid effect of promethazine. Propranolol itself induced an antiplasmid effect. The effects of beta-receptor agonists and antagonists on promethazine-induced antiplasmid action serve as an indirect evidence of beta adrenergic like binding sites in *E. coli*. These binding sites are involved in the plasmid replication process and are connected with promethazine binding sites in bacteria.

Antibacterial and antiplasmid effects of various phenothiazines and structurally related tricyclic drugs have been demonstrated on both Gram-positive and Gram-negative bacteria and even mycobacteria [1–6]. These compounds had membrane effects [5, 7] and were able to intercalate into the DNA [8]. It was presumed that they may decrease the active transport of amino acids and the electrochemical proton gradient or proton pump in the bacteria [9].

Chlorpromazine and amitriptyline are non-competitive inhibitors of the N-methyl-D-aspartate (NMDA) receptor [10]. The two drugs have both antibacterial and antiplasmid effects [7]. It has therefore been assumed that both act on bacteria via this membrane receptor similar to NMDA. If this is true it can be expected that some of the excitatory amino acids that are able to modify the NMDA receptor will prevent the antibacterial or antiplasmid action of tricyclic drugs such as promethazine. The strict competition of promethazine with excitatory amino acids could be excluded. Phenothiazines may inhibit amino acid transport, which is driven by proton

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electrochemical gradients [9]. The membrane then becomes hyperpolarized [11] and as a consequence the calcium influx and potassium efflux are increased [12] and the dopamine D₁ receptor-mediated functions are blocked [13]. Although other dopamine receptors mediate the action of phenothiazines [14, 15], some of these receptors undergo changes in certain diseases [16, 17].

To clarify the mechanism of action and binding of phenothiazines in bacteria, we have measured the growth rate of *E. coli* and the F⁺lac plasmid elimination of promethazine in the presence of various amino acids, or dopamine, phenylalanine derivatives and some beta-receptor agonists and antagonists. We assumed that one binding site of phenothiazine in bacteria are related to one of the known receptors of drugs or amino acid uptake mechanism and can be inactivated by promethazine or some of the beta-receptor antagonists.

Here we report that some amino acids and structurally related compounds such as beta-receptor agonists restore biochemical pathways essential for plasmid replication and prevent the antibacterial and antiplasmid effects of promethazine.

Materials and methods

Chemicals. The beta-receptor agonists isoproterenol and antagonists such as propranolol, oxyprenolol and alprenolol isomers and phenoxybenzamine (alpha-adrenergic antagonist) were produced by Sigma. Promethazine (Pipolphen®) was produced by EGIS Pharmaceutical Works (Budapest). Amino acids were produced by Reanal (Budapest/see Tables I and II).

Bacterial strain. *E. coli* K12 LE 140 F⁺lac was provided by Professor I. B. Holland (Department of Genetics, University of Leicester, UK).

Culture media. M-9 minimal medium and MTY broth were prepared according to Alföldi et al. [18]. Eosine methylene blue agar (EMB) was used for the detection of plasmidless colonies [19].

The antibacterial effect and elimination of F⁺lac plasmid were tested as described by Molnár and Mándi et al. [3, 4] in M-9 minimal medium [20].

Results

Promethazine has antibacterial and antiplasmid effects. Because of its unique biological effects, its antibacterial and antiplasmid actions were studied in the presence of different amino acids, including excitatory ones, to establish whether they reverse the biological effects. It was found that glutamic acid, aspartate, glycine, proline, threonine, histidine and kainic acid partially prevented only the antibacterial action of phenothiazine in MTY broth (data not shown).

In complete media MTY broth promethazine at 90 µg/ml had an antiplasmid effect in 55–60% which was not altered in the presence of glutamate, aspartate, glycine, proline, threonine, histidine, kainic acid, tryptophan, leucine, isoleucine or methionine. Although glycine, threonine and kainic acid reduced the antiplasmid effect.

Table I

Antiplasmid action of promethazine in the presence of amino acids of E. coli F⁺lac

Amino acids 250 µg/ml	Viable cells × 10 ⁻⁸	Lac ⁻ colonies %
Control	0.76	50
Alanine	0.79	20
Glycine	0.86	4.6
Leucine	0.68	29
Isoleucine	0.68	36
Gamma-aminobutyric acid	0.75	18
L-phenylalanine	1.30	0-1
D-phenylalanine	1.20	0-4
DOPA	0.06	0.4-4
Phenylpyruvic acid	0.82	1-9
Phenylacetic acid	0.80	1-10
Proline	0.89	30
Valine	0.74	28
Para-aminobenzoic acid	0.96	30
Cysteine	0.70	25
Cystine	0.75	27
Methionine	0.72	40
Serine	0.78	5.8
Threonine	0.90	7.2
Tyrosine	0.85	0.2-2.4
Tryptophan	0.70	30
Aspartate	1.90	40
Glutamate	1.20	40
Kainic acid (50 µg/ml)	0.80	20
Arginine	1.20	28
Histidine	0.73	39
Lysine	0.98	37
Noradrenaline	0.70	1

Amino acids were applied in 250 µg/ml and promethazine in 40 µg/ml final concentration in M-9 minimal medium and the samples were incubated for 48 h at 37 °C

In M-9 minimal medium, promethazine at 40 µg/ml caused F⁺lac plasmid elimination with 50% frequency, which was somewhat reduced in the presence of 250 µg/ml glycine, phenylalanine, phenylpyruvic acid, phenylacetic acid, serine, threonine or tyrosine and noradrenaline (Table I). At 60 µg/ml promethazine the bacteria were not able to grow. There was little difference between the reversal effects of L- and D-phenylalanine on bacterial plasmid replication in minimal M-9 medium. Tyrosine, dopamine, phenylacetic acid and phenylpyruvic acid had a similar inhibitory action on the plasmid curing effect of promethazine. The effect was concentration dependent (Table II). As a consequence of oxidation, dopamine produced a brownish discoloration in the medium after 3-5 h, which prevent the antiplasmid effect. Agonists e.g. isoproterenol, noradrenaline (and phenoxybenzamine) and antagonists (e.g.

propranolol, oxyprenolol and alprenolol isomers) were also tested on the antiplasmid effect of promethazine in subsequent experiments. As Table III shows, the non-selective beta-agonists lowered, while the antagonists enhanced the antiplasmid effect of promethazine in MTY broth. The beta-antagonists tested alprenolol and oxyprenolol were not able to eliminate the F'lac plasmid of *E. coli*, however, propranolol itself had a moderate antiplasmid effect in *E. coli* cultures.

Table II

Plasmid curing effect of promethazine (40 µg/ml) in the presence of DOPA, tyrosine or noradrenaline in M-9 minimal medium

Compounds µg/ml	Bacterial growth density 48 h	Plasmidless lac ⁻ colonies %
Promethazine (40 µg/ml)		
+ DOPA		
1 µg/ml	0.25	25
4	0.23	15
5	0.28	9
10	0.30	4
+ Tyrosine		
50 µg/ml	0.17	25
300	0.20	23
400	0.22	11
500	0.30	4
+ Noradrenaline		
0.5 µg/ml	0.20	19
1	0.02	6.5
6	0.06	0.9
8	0.07	4.2
10	0.22	0.5
+ Promethazine		
0 µg/ml	0.40	0
40	0.03	19

Discussion

The reversal by dopamine of the antibacterial effect of promethazine suggested a possible competition between dopamine and promethazine. Such binding may occur through charge transfer complexes. Indeed, it has been shown that phenylalanine, dopamine and other neurotransmitters can form charge transfer complexes with phenothiazines and related drugs [21, 22]. Even the oxo derivative of dopamine has antibacterial effects [6]. The brownish discoloration developing in the bacterial culture suggested an intramolecular charge transfer. Chlorpromazine and promethazine form charge transfer complexes with an L-phenylalanine derivative [23] and other compounds [24]. Phenylalanine derivatives may have a regulatory role and may act as signal transmitters in bacteria.

Table III

Plasmid curing action of promethazine (60 µg/ml) in the presence of adrenergic agonists and antagonists on E. coli F⁺lac in MTY broth

Compounds	Concentration µg/ml	Lac ⁺ plasmidless cells in per cent	Viable cells/ml after 24 h, ×10 ⁷
DL-Isoproterenol	0	35	1.9
	10	36	1.6
	20	35	1.2
	40	22	0.9
	80	16	1.9
	100	12	1.8
	120	6	1.4
Phenoxybenzamine	0	44	0.3
	10	37	0.3
	20	20	0.5
	40	3	0.4
	60	0.5	0.1
Propranolol (DL)	0	32	2.4
	10	51	2.1
	20	55	3.0
	40	61	2.0
Oxyprenolol (Trasicor)	10	30	1.1
	20	40	1.3
	40	50	1.9
	60	60	3.8
	80	55	3.0
	100	66	4.0
	200	0	4.2
D-Alprenolol	0	34	2.9
	10	47 28.5	2.4
	20	58	2.2
	40	76 57.5	1.5
	60	—	—
	80	—	—
	100	—	—
	200	—	6.4
L-Alprenolol	0	34	3.4
	10	35 2.0	3.5
	20	42	2.0
	40	55 36.9	2.1
	60	69	1.8
	80	83	1.5
	100	64.7	—
Propranolol (without promethazine)	40	0	19.0
	80	0	3.5
	150	2.1	2.9
	200	12.1	0.8

The phenothiazine complex may lose the biological activity, which accounts for the suppression by the above compounds of the antibacterial and antiplasmid effect of promethazine.

At a receptor level, there are also possibilities for interaction between promethazine and receptor substances. The bound promethazine molecule occupies the binding site which is essential in the signal transduction for bacterial growth or plasmid replication. However, the natural substrate of the binding site can compete with phenothiazine. Noradrenaline and isoproterenol beta-receptor agonists reduce the effects of promethazine if they are bound to the same receptor or binding site. In reversal of the antiplasmid effect it can be the case, since beta adrenergic receptor antagonists enhance the antiplasmid effect of promethazine. It is presumed that binding sites exist in bacteria and they are involved in the action of promethazine.

Circumstantial evidence confirms the above hypothesis: propranolol alone (adrenergic receptor antagonist) can induce the antiplasmid effect. Phenoxybenzamine alpha adrenergic antagonist reduce the sensitivity of beta adrenergic binding site [25–27]. Our experimental data led us to suppose that beta-receptor like structures can exist and may play a regulatory role in plasmid replication in *E. coli*. Nevertheless, the nature of the adrenergic binding sites in bacteria needs further investigations.

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STUDIES ON THE EXISTENCE OF SYNERGISM BETWEEN DIFFERENT ANTIBIOTICS AND A PHENOTHIAZINE TRANQUILLISER, PROMAZINE, POSSESSING ANTIMICROBIAL PROPERTY

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Different antibiotics were tested for their capacity to exhibit synergism when used in combination with promazine (Pr), a tranquiliser endowed with powerful antimicrobial property. For this purpose the minimum inhibitory concentration (MIC) of different antibiotics and Pr was first determined by spot inoculation technique on nutrient agar plates to select the concentrations of the antibiotics and Pr to be used as well as the sensitive strains. It was observed that Pr in combination with tetracycline (Tc) demonstrated a marked enhancement of the inhibitory capacity of each drug, both against the Gram-positive and Gram-negative bacteria, following disc diffusion technique. The *in vitro* findings were further substantiated with the *in vivo* effects of Pr along with Tc using *Salmonella typhimurium* NCTC 74 as the challenge strain in mice. The synergism obtained was further corroborated in terms of the increase in the size of their inhibition zones compared with their unaltered individual zones to determine the level of significance. This result was also confirmed by the checkerboard test using doubling dilutions of both the agents.

A large number of drugs possessing different pharmacological activities have been reported by various worker to also exhibit distinct antimicrobial activities. Our earlier reports include antimicrobial activities of several antihistaminic drugs namely bromodiphenhydramine and diphenhydramine [1, 2], tripolidine [3], methdilazine [4, 5], some antihypertensive drugs such as methyl-DOPA [6] and propranolol [7] as well as the tranquiliser drug promazine [8]. Such a study has been further substantiated by the studies of Kristiansen [9] and Molnár et al. [10] who have demonstrated the

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antimicrobial activity and the antiplasmid activity of these non-antibiotics which comprise mainly the phenothiazines. This antimicrobial effect could be further substantiated upon suitable structural modifications and also by discovering their effect of combined action with different antibiotics. Hence such a search could become a subject of interesting study from the point of view of their mutual synergism, indifference or antagonism [11, 12]. In present communication we report on the existence of synergistic reaction between the tranquiliser promazine (Pr) and several antibiotics.

Materials and methods

Drugs. The antibiotics used were penicillin (Pc), ampicillin (Ap), streptomycin (Sm), kanamycin (Km), chloramphenicol (Cm), erythromycin (Er), tetracycline (Tc), dimethylchlorotetracycline (DMCT) and the chemotherapeutic agent promazine (Pr). These agents were obtained from the respective manufacturers and were stored at 4 °C in dry powder form.

Strains. The strains employed in this study along with their sources are described in Table I.

Media. The liquid media used in the study were peptone water consisting of 1.0% bacteriological peptone (Oxoid) plus 0.5% NaCl (Analar) and Mueller-Hinton broth (Oxoid). The solid media used were obtained by solidifying the above-mentioned liquid media with 1.0% agar (Oxoid No. 3). Blood agar was prepared by adding 10% sterile defibrinated sheep blood to a nutrient agar base. The Gram-negative organisms were grown in peptone agar while Mueller-Hinton and blood agar were used for the other organisms.

Determination of the Minimum Inhibitory Concentration (MIC) of Pr and other antibiotics. Nutrient agar plates containing 1, 2, 5, 10, 20, 25, 50, 100, 200, 500, 800 and 1000 µg of the agents (Pr, Pc, Ap, Sm, Km, Cm, Er, Tc and DMCT) were spotted in triplicate with about 10⁵ colony forming units (c.f.u.) of different bacteria and checked for growth upto 48 h with extended incubation when necessary. This agar dilution technique [13] was carried out for accurately determining the MIC of Pr, Pc, Ap, Sm, Km, Cm, Er, Tc and DMCT with respect to various bacteria.

Method for assessment of combination of antibacterial effects of Pr with other agents. For this purpose the diameters of the zones of inhibition produced individually by each of a pair of agents were determined first following disc diffusion technique [14]. Based on these results, the test for combination was performed by placing the discs in such a manner that their inhibition circles (e.g. those of Pr and Tc) would touch each other tangentially if the reaction between them was one of indifference only. Inhibition circles which receded from each other showing concavity towards each disc, clearly indicated antagonism, however, in cases of synergism the inhibitory circles increased in size (as in Fig. 1). Here the diameters of the inhibition circles produced under mutual influence were determined as against those due to their individual (uninfluenced) zones. The increase in the surface area (πr^2) if any, due to the combination vis-à-vis those due to single effects, was statistically evaluated for the level of significance. The effects observed due to the single effects of these agents were further examined by the "checkerboard" dilution test for corroboration as well as for determining the fractional inhibitory concentration (FIC) index and the minimum bactericidal concentration (MBC) according to Krogstad and Moellering [15] using doubling dilutions of the pair of the test agents in Mueller-Hinton broth (Fig. 2).

In vivo antibacterial tests. For the in vivo studies a Swiss strain of white mice weighing 15–18 g were used and these were maintained in cages with pellet food and water in our animal house. The challenge strain was *S. typhi-murium* NCTC 74 as it was naturally virulent to mice and also sensitive to Pr and Tc in vitro. The virulence of this strain was increased by repeated mouse passages by administering intraperitoneally and recovering from the heart blood. In order to determine the median

lethal dose (MLD or LD₅₀) of this challenge strain, it was administered in increasing amounts to batches of mice, which were observed for 100 h and the mortality recorded. This strain was then preserved by freeze drying and reconstituted when necessary. Standardization of the optical density of the challenge dose at 640 nm in a Klett Summerson Colorimeter ensured reproducibility of the same dose for each use. Then Pr and Tc were administered at approximately 1.0 µg/g and 4.5 µg/g body weight doses intraperitoneally as 0.1 ml sterile solutions into a mouse, 3 h before the challenge.

For determining the effect of *in vivo* combinations of Pr and Tc, eight batches of animals each batch having six mice were all given the challenge dose, two batches received either Pr or Tc, (1.0 µg/g or 4.5 µg/g of mouse, respectively) 3 h before the challenge, while the other two batches received a combination of Pr (1.0 µg/g) and Tc (4.5 µg/g), and the remaining two batches received only saline. All the animals in one batch were autopsied 2 h or 18 h after the challenge. Their livers and spleens were removed and homogenized in sterile glass homogenizers under aseptic conditions and preserved at -20 °C for determination of c.f.u./ml. From these animals 0.2–0.4 ml amount of heart blood samples were also collected at the same time, allowed to clot and analyzed for the size of bacteraemia (by clot culture) and drug concentration in the sera. The drug concentration was also determined at 0 h in all the mice in a separate experiment.

Results

Minimum Inhibitory Concentration (MIC) of different antibiotics and Pr. *Staphylococcus aureus*, *S. typhi-murium*, *Shigella boydii* and *Vibrio cholerae* (Table I) were used for this purpose. With respect to *S. aureus* the MIC of all the antibiotics existed between 5 and 20 µg/ml level. However, the MIC of Pr in case of all the three strains was 25 µg/ml. For *S. typhi-murium* and *S. boydii* the MIC of the antibiotics ranged from 8 to 15 µg/ml, and that of Pr was 50 µg/ml and 25 µg/ml, respectively. The range of MIC of the antibiotics was slightly broader with respect to *V. cholerae* (Table I). Only the highly sensitive strains were used in further experiments.

Effects of combination of activities of Pr with Tc. Table II reveals that the inhibition zones due to combined effects of Tc and Pr were 17.3 and 19.6 mm, as compared to 16.1 and 17.8 mm, respectively, when tested singly against *S. aureus* ML 160. It was also noted that in case of *S. aureus* NCTC 8530 combination of Tc and Pr produced a marked enhancement of the inhibitory capacity of each drug. On testing for synergistic effects of Pr with Tc against different Gram-negative bacteria (Table II) marked synergism was also observed. The synergism observed between Tc and Pr in terms of per cent increase in the size of their inhibition zones was assessed for their level of significance for both the agents (Table III). The activity of Tc was 15.5 and 16.9% greater in respect of Gram-positive bacteria while 5.6 and 6.2% greater in respect of Gram-negative ones in comparison with their individual effects. Similarly, the activity of Pr was 21.25% and 23.5% higher in case of the Gram-positive bacteria while the activity increased by 6.78% and 31.5% with respect to the Gram-negative strains. The per cent increase in the surface area for Pr and Tc with respect to *S. aureus* was found to be significant. This effect was further confirmed by the checkerboard test using doubling dilutions (µg/ml): 20 to 0.31 against Tc and 100 to 1.56 with Pr. The MIC (µg/ml) of Tc and Pr singly were 10 and 50 while in combination were 1.2 and 12.5 or lower. The FIC index for *Escherichia coli* C21 (not listed in Table I) was found to be 0.37 which again confirmed the presence of synergism.

Table I

Minimum inhibitory concentration (MIC) of various antibiotics and promazine with respect to the test bacteria.

Strain	Minimum inhibitory concentration (MIC) µg/ml									Source
	Pc	Ap	Tc	DMCT	Cm	Er	Sm	Km	Pr	
<i>Staphylococcus aureus</i> 8530	5	5	5	10	5	10	10	10	25	S.P. Lapage, London
<i>S. aureus</i> 8531	5	5	5	10	5	10	10	10	25	S.P. Lapage, London
<i>S. aureus</i> ML	10	10	10	10	10	25	10	20	25	M. Lahiri, Calcutta
<i>Salmonella typhi-murium</i> 74	8	8	10	10	8	10	8	8	50	J. Taylor, London
<i>Shigella boydii</i> 8254/66	5	5	5	10	15	10	10	10	25	K.P. Carpenter, London
<i>Vibrio cholerae</i> 540		20	8	10	8	10	4	2	100	S. Mukerjee, Calcutta
<i>V. cholerae</i>	4	8	50	50	2	10	8	8	100	S. Mukerjee, Calcutta

Pc, penicillin; Ap, ampicillin; Tc, tetracycline; DMCT, demethyl chlortetracycline; Cm, chloramphenicol; Er, erythromycin; Sm, streptomycin; Km, kanamycin; Pr, promazine.

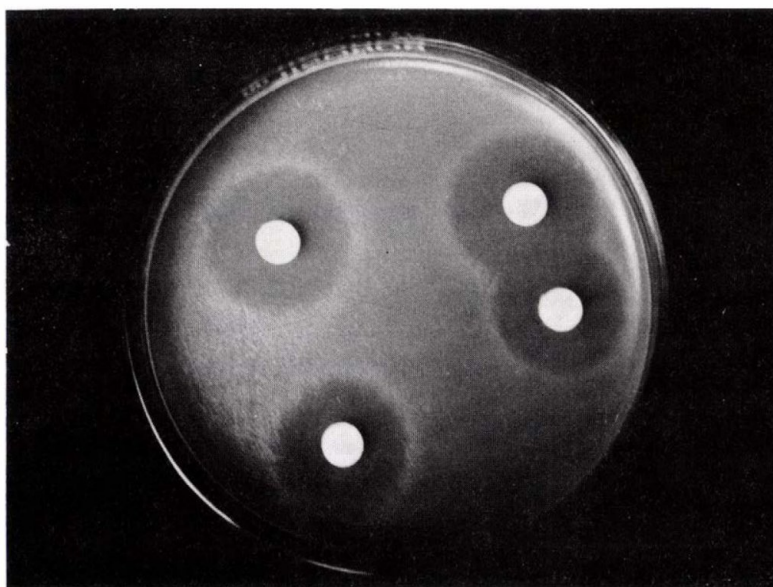


Fig. 1. Synergism between Pr and Tc against *S. typhi-murium* 74 on peptone agar

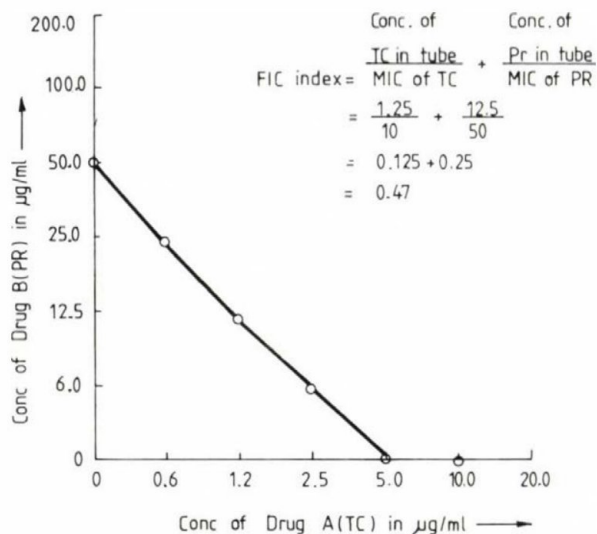


Fig. 2. Isobologram plotted from results of checkerboard test

Determination of in vivo activity. The median lethal dose (LD_{50}) was determined for the strain *S. typhi-murium* NCTC 74, based on the mortality ratio data (Reed and Muench, 1938] and was found to be 1.8×10^7 c.f.u./ml. The in vivo studies of the blood and organ homogenates of 24 h normal mice belonging to the same stock as in the experimental animals yielded no *S. typhi-murium* 74 or other salmonellae. Promazine (2.25 µg/g of mice) along with Tc (6 µg/g of mice) significantly reduced the c.f.u./ml counts of bacteria in organ homogenates of animals 2 and 18 h after challenge as compared with the control ($P < 0.01$) for 2 and 18 h liver samples and $P < 0.05$ for all others (presented in details in Table IV and Table V).

Discussion

From the present investigation we have been able to prove the existence of synergism between Pr and Tc with respect to several sensitive Gram-positive and Gram-negative bacteria, on the basis of disc diffusion tests. Quantitative estimation of the disc diffusion test, by employing per cent increase in surface area of the individual inhibition zones compared to the ones produced by combined effect showed a significant enhancement of antibacterial property. The activity of Tc was higher with respect to both Gram-positive and Gram-negative bacteria in contrast to their individual effects. The action of Pr also increased. The in vitro synergistic activity was also found to be highly valid statistically.

Table II

Inhibitory effects of tetracycline and promazine singly and in combination on different bacteria

Strain	Zone of inhibition in mm*					
	Individual effect (µg/ml)		Combined effect (µg/disc)		Increase (mm) Combined - Individual	
	Tc (10)	Pr (200)	Tc (10)	+ Pr(200)	Tc	Pr
<i>Staphylococcus aureus</i> ML 160	16.1	17.8	17.3	19.6	1.2	1.8
<i>S. aureus</i> NCTC 8530	16.0	18.0	17.3	20.0	1.3	2.0
<i>S. aureus</i> NCTC 8531	14.0	16.0	14.6	16.5	0.6	0.5
<i>Salmonella typhi-murium</i> 74	15.0	16.6	15.3	16.8	0.3	000.2
<i>Shiaella boydii</i> 8 254/66	18.0	18.0	18.5	18.6	0.5	0.6
<i>Vibrio cholerae</i> ATCC 14033	13.0	15.0	13.4	17.2	0.4	2.2
<i>V. cholerae</i> 540	16.0	15.9	16.3	16.6	0.3	0.7

Tc, tetracycline; Pr, promazine

* Calculated as the average of triplicate measurements

Table III

Increase (%) in effects of Tc and Pr compared with their individual activities based on surface area determination of their inhibition zones.

Strains	Mode of action	Inhibition due to Tc in terms of		Inhibition due to Pr in terms of	
		Diam in mm (2r)	Surface area (πr^2)	Diam in mm (2r)	Surface area (πr^2)
<i>S. aureus</i> ML 160	Singly	16.1	A) 203.61 mm ² *	17.8	A) 248.88
	In combination	17.3	B) 235.09 mm ²	19.6	B) 301.76
	% Increase in surface area		15.46% **		21.25%
<i>S. aureus</i> NCTC 8530	Singly	16.0	A) 201.08 mm ²	18.0	A) 254.50 mm ²
	In combination	17.3	B) 235.09 mm ²	20.0	B) 314.20 mm ²
	% Increase in surface area		16.91%		23.46%
<i>S. boydii</i> 8 254/66	Singly	18.0	A) 254.50	18.0	A) 254.50 mm ²
	In combination	18.5	B) 268.84	18.6	B) 271.75 mm ²
	% Increase in surface area		5.63%		6.78%
<i>V. cholerae</i> ATCC 14023	Singly	13.0	A) 132.75	15.0	A) 176.74 mm ²
	In combination	13.4	B) 141.05	17.2	B) 232.38 mm ²
	% Increase in surface area		6.25%		31.48%

* mean surface area of the inhibition zone (mm²) was calculated as πr^2 on the basis of measurement of its mean diameter (2r)

** % increase calculated as B-A/Ax100

Table IV

Viable counts (c.f.u./ml) of *S. typhi-murium* 74 of organ homogenates

Time of sampling	Mouse No.	Drug ($\mu\text{g/g}$ of mouse)	c.f.u./ml counts in	
			Liver	Spleen
2 h	1	Tc (6 $\mu\text{g/g}$ of mouse)	4.0×10^4	2.0×10^4
	2		2.0×10^4	2.6×10^4
	3		2.3×10^4	3.0×10^4
	4		3.6×10^4	4.2×10^4
	5		4.6×10^3	4.8×10^3
	6		3.8×10^4	3.0×10^4
	1	Pr (2.25 $\mu\text{g/g}$ of mouse)	3.6×10^4	3.4×10^5
	2		4.0×10^4	2.3×10^4
	3		4.6×10^4	2.6×10^4
	4		4.3×10^4	2.8×10^4
	5		4.8×10^4	3.2×10^4
	6		4.7×10^4	4.2×10^5
	1	Tc (6 $\mu\text{g/g}$ of mouse) + Pr (2.25 $\mu\text{g/g}$ of mouse)	1.3×10^3	1.6×10^3
	2		6.6×10^2	3.6×10^3
	3		1.6×10^3	3.2×10^3
	4		6.0×10^2	7.4×10^3
	5		2.3×10^3	8.8×10^3
	6		3.6×10^3	7.6×10^3
	1	Saline Control	4.0×10^6	5.1×10^5
	2		3.6×10^6	4.3×10^6
	3		3.4×10^6	7.6×10^6
	4		4.3×10^6	4.1×10^6
	5		4.6×10^5	3.8×10^6
	6		3.3×10^6	4.6×10^6

All the mice received a challenge dose of 9.0×10^9 of *S. typhi-murium* 74 and the drug(s) were administered 3 h before the challenge. The mice were killed 2 h after the challenge and their liver and spleen homogenates were prepared for viable counts. When viable counts of 2 h liver and spleen with respect homogenates to Pr and Pr with Tc were compared "t" test with the control, $P < 0.01$ and thus highly significant.

Hence the powerful antibacterial activity possessed by the non-antibiotic phenothiazine compound Pr coupled with its synergistic response with Tc suggests that Pr like many other antibacterial chemotherapeutic agents can effectively combat many infections caused by Gram-positive and Gram-negative bacteria.

This synergistic combination of a conventionally "non-microbial" drug with an antibiotic provides scopes for scrutiny and identification of newer combinations. In the outcome we may stand to gain not only a new synergistic combination for antimicrobial therapy, but one such that possesses an additional dimension of a desirable function, i.e. of tranquilising and sedative reactions, a necessity that is so common with the use of various antimicrobial drugs with or without synergistic effects.

Table V

Viable counts (c.f.u./ml) of *S. typhi-murium* 74 of organ homogenates

Time of sampling	Mouse No.	Drug ($\mu\text{g/g}$ of mouse)	c.f.u./ml counts in	
			Liver	Spleen
	1	Tc (6 $\mu\text{g/g}$ of mouse)	8.0×10^5	5.0×10^5
	2		8.1×10^5	4.5×10^5
	3		7.0×10^5	4.0×10^6
	4		6.5×10^5	5.2×10^6
	5		6.0×10^5	5.8×10^5
	6		7.0×10^6	6.5×10^5
	1	Pr (2.25 $\mu\text{g/g}$ of mouse)	6.0×10^6	5.0×10^6
	2		7.0×10^6	4.5×10^6
	3		5.5×10^6	5.2×10^6
	4		6.5×10^6	4.2×10^6
	5		4.5×10^7	4.0×10^7
	6		5.6×10^7	2.6×10^6
	1	Tc (6 $\mu\text{g/g}$ of mouse) + Pr (2.25 $\mu\text{g/g}$ of mouse)	2.0×10^3	3.5×10^4
	2		4.0×10^4	2.6×10^4
	3		3.8×10^4	3.6×10^4
	4		5.0×10^4	4.6×10^4
	5		6.0×10^4	4.8×10^4
	6		4.5×10^4	6.2×10^4
	1	Saline Control	2.5×10^7	4.0×10^7
	2		4.5×10^6	4.2×10^6
	3		5.0×10^6	4.6×10^6
	4		4.2×10^6	3.6×10^6
	5		11.0×10^6	5.6×10^6
	6		5.0×10^7	4.8×10^7

All the mice received a challenge dose of 9.0×10^9 CFU of *S. typhi-murium* 74, 3 h after administration of the drugs. The mice were killed 18 h after challenge and their livers and spleens were homogenised and checked for viable bacteria. Statistical analysis of the data of combined effect (Pr + TC) when compared by "t" test with the control was $P < 0.01$.

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SUPPRESSED PHAGOCYTOSIS BUT PROMOTED BACTERIAL GROWTH UPON THE EFFECT OF VIRUS FREE SUPERNATANTS OF INFECTED LYMPHOCYTES

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The pathomechanism of suppressed phagocytosis and bacterial superinfections which follow viral diseases is not completely understood. Both polymorphonuclear leukocytes (PMNL) and mononuclear phagocytes (MNPh) as well as bacterial growth are controlled by several cytokines produced by other immune cells. The effect of disturbed production of cytokines on phagocytes and microbes have not been studied yet. Peripheral T lymphocytes were infected with human adenoviruses (Ad) and herpes simplex virus type 1 (HSV-1) or were activated by phytohaemagglutinin (PHA), then culture supernatants containing no infectious virus were mixed to phagocytes swallowing viable *Staphylococcus aureus*. Influence of supernatants on eukaryotic and bacterial cell growth was compared to cytokine assays. Supernatant mediators, different from interferon (IFN) and tumour necrosis factor (TNF), induced by oncogenic Ad-12 or HSV-1 diminished phagocytosis of both PMNL and MNPh in a dose dependent manner, promoted bacterial growth free and inside MNPh, while those of latent Ad-5 and nononcogenic Ad-8 exerted moderate effects. Plating efficiency of HEp-2 cells was decreased by all of them. Supernatants of PHA treated lymphocytes containing IFN- γ enhanced both phagocytosis and bacterial replication free and inside MNPh, while it suppressed HEp-2 plating. Neither viruses nor PHA affected phagocytic process

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directly. Staphylococci inside PMNL were not affected. Presumably, production of a single mediator by infected T lymphocytes is responsible for the multiple effects. Relationship with another soluble factors is discussed.

Immunosuppression induced by viruses and consequent frequent microbial (bacterial, fungal) superinfections have gained clinical importance with acquired immunodeficiency syndrome (AIDS) [1]. Not only its causative agent, human immunodeficiency virus type 1 (HIV-1), but several other viruses belonging to different virus groups can induce acute or chronic immunosuppressions, although their pathomechanisms vary widely [2-4]. The most obvious way that a virus may suppress immune functions is by replicating in and destroying the cells which carry out that function. Another possible way is the activation of suppressor cells. Some virion components also have direct suppressive effect on well defined immune functions [1]. These do not explain phenomena why such immune functions are diminished whose effector cells do not serve as target or site for infection. These immune cells execute non-specific defence functions as intracellular killing of invading microorganisms by polymorphonuclear leukocytes (PMNL) or mononuclear phagocytes (MNPh), or destroy infected cells via extracellular mechanisms by natural killer (NK) cells. Phagocytosis is the most important defence mechanism against invading bacteria and fungi [5], but its role has long been underestimated in antiviral defence: one PMNL or MNPh is capable of internalizing thousands of virus particles, e.g. influenza, vaccinia, type 1 herpes simplex virus (HSV-1). Most viruses are not able to replicate inside PMNL and are never found in the cytoplasm or nucleus [6]. Replication of viruses, e.g. HSV-1 or HIV-1 in macrophages is related to the state of their differentiation [6], and with viruses that are unable to multiply in them, macrophages appear to play their usual role as scavengers [8].

The importance of phagocytes to health is dramatically illustrated by the disastrous effect of reduction in their number [8-11] or inadequate functions [5] in virus infections. Infection by HIV-1 or feline immunodeficiency virus (FIV) of both PMNL and MNPh, or that of by Newcastle disease virus (NDV), measles, influenza of PMNL can suppress their functional capacity directly, but infection of cat PMNL by UV-treated feline leukaemia virus (FeLV) also decreased phagocytic activity in spite of 98% viability of cells [12]. Others established that, depressed PMNL functions by NDV, FeLV, measles, Epstein-Barr virus (EBV) were not dependent on active infection, rather a possible T cell defect causing PMNL depletion in virus infected host [13]. Infection of rabbits with HSV-1 resulted in almost total inhibition of phagocytosis by alveolar and peritoneal macrophages, whereas only about 40% of them became infected with the virus. Possibly, impairment of phagocytosis was the result of virus-mediated toxicity which occurred independently of viral replication [7]. The pathomechanism of the above phenomena has not yet been clarified.

The life cycle and phagocytic process of both PMNL and MNPh are regulated positively or negatively by several cytokines released by another immune cells, especially T lymphocytes [14-17]. Furthermore, both phagocyte types are able to produce several soluble mediators which influence each other or another immune cells [14-16, 18]. Phagocyte maturation from common stem cells are promoted by

granulocyte-monocyte colony stimulating factor (GM-CSF), granulocyte (G-CSF), and monocyte (M-CSF), [10, 14, 19]. Transendothelial migration and chemotaxis are regulated by interleukin 8 (IL-8) and leukotriene B₄ (LT-B₄) [14]. Interferon- γ (IFN- γ) produced mainly by activated CD4 T lymphocytes [16] enhances the capacity of both PMNL and MNPh to produce reactive oxygen and nitrogen intermediates (ROI, RNI) as well as granule constituents, to induce expression of surface receptors important in signal transduction during particle engulfment [15, 16, 19]. IL-3 produced by activated T cells stimulate both phagocytes, tumour necrosis factor- α and - β (TNF- α and β), augment phagocyte and killing activities of cellular microbes [15] and augment superoxide anion (O₂⁻) production [19]. TNF- α and GM-CSF have been reported to enhance phagocytosis, productions of ROI, RNI, LT and IL-1 by PMNL [20, 21]. IL-4 depresses respiratory burst capacity and antimicrobial activity of macrophages, while oxygen metabolism, antimicrobial activity and IL-8 production by PMNL were found to be unaffected [16]. Transforming growth factor β_1 (TGF- β_1) inhibits PMNL-endothelial cell adhesion augmented in vitro by TNF- α , but TGF- β_1 can prime tissue sites for enhanced PMNL recruitment in response to subsequent inflammatory stimuli [18]. Both TGF- β_1 and - β_2 and a macrophage deactivating factor (MDF) can inhibit respiratory burst capacity of MNPh [21].

Moreover, MNPh treated with IFN- γ blocks the growth of intracellular pathogens, whose growth they support in its absence possibly by elevated RNI production [19]. It has recently been demonstrated that some cytokines (IL-1, IL-2, GM-CSF, TNF- α) can bind to receptors on different bacteria and fungi enhancing their growth and modifying phagocytic uptake or cellular invasion [22]. Furthermore, most cytokines function pleiotropically [23]: receptors for several interleukines have recently been found on most somatic cells, and therefore they are able to moderate growth of normal and tumourous cells: IFN- γ , IL-5, oncostatin all produced by activated T cells, as well as IL-6, TGF- β and TNF- α and - β are well known for their inhibitory effects on tumour cells [19].

As the importance of T cell-phagocyte interaction is slowly appreciated [8, 15, 19], disturbed mediator release by virus infected lymphocytes with consequent alterations in lymphocyte-target cell population becomes conceivable. Suppressor factors elaborated by infected lymphokines, macrophages or non-immune cells have been described displaying a broad range of molecular weights and activities [1]. The EBV encoded protein, BCRF1 possesses striking structural similarity to IL-10, which latter is produced by T_h 2 cells and suppresses cytokine production by T_h 1 cells. BCRF1 also exhibits the inhibitory activity of IL-10 on the synthesis of other cytokines [24]. Normal human T cells do not produce IL-1 or IL-6, but adult T cell leukaemia (ATL) line (their ethiology is linked to human T lymphotropic virus type I, HTLV-I) produce IL-1. HTLV-I infection of T lymphocytes induces constitutive production of IL-6. HIV-1 infected AIDS patients show high serum concentrations of IL-6 [23]. Poxviruses codify for a viral growth factor and for other virokinines, while vaccinia virus induces unusual selective expression of IL-2, IL-2Ra and IL-6, but not IFN- γ in lymphocytes [25]. Suppressor factors are also frequently produced by virus-transformed tumour cells [1]. TGF- β was originally described as a product of murine

sarcoma virus transformed cells. Soluble factors of tumour bearing individuals also inhibit functions of macrophages and other immune cells [26]. A factor was produced by peripheral blood mononuclear cells (PBMC) of human papillomavirus (HPV) infected persons, whose cells were grown in RPMI with or without the presence of serum for several days. Supernatants in their crude form or after filtration or different treatments with heat, acid, proteolytic enzymes or eluting with different solvents from chromatography columns were added to T-lymphocytes separated through nylon wool columns. Viability and specific function of cells were monitored. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie or silver staining was used to estimate molecular size and composition of factors [2, 26]. Dialysis before bioassays is used to eliminate low molecular weight products of cells, mainly prostaglandins (PG) and LTs, which also have antiphagocytic and immunomodulatory activities [2, 27]. The effect of these factors on various immune functions have not been explored.

Among DNA viruses which frequently infect T lymphocytes, adenoviruses (Ad)[28–31] and HSV-1 [32–34] are important human pathogens with potentials of immunosuppression and latency [31, 35–37]. This latter means that in spite of frequent virus carriage, the replication of Ad [38, 39] and HSV-1 [32, 34] is very limited in resting blood lymphocytes. Consequences of Ad infection in immune cells, especially lymphokine production have already been studied in part: human Ads are described IFN inducers [40]. This activity is weak in human cells but potent in chick cell cultures or in hamsters after intravenous inoculation [1]. The mechanism and quantity of IFN production show correlation with oncogenic potential of adenovirus serotypes: highly oncogenic types 12, 18, 31 (Group A) and type 8 of Group D induce high amount of IFN, while latent types of Group C including Ad-5 or Ad-2 induces low level IFN [41–43]. Low infectivity in human cells is a striking common characteristics of the effective inducers, while types having higher titres are less effective inducers [43]. Incomplete particles of type 12 Ad [42] found in nonpurified virus stocks augment IFN production [43]. In contrast, another cellular product with protective effects, 22 and 27 kD heat shock proteins were expressed only in nononcogenic adenovirus transformed cells and untransformed cells [44].

Infection by HSV-1 may involve the production of lymphokines: two types of leukocyte migration inhibitory factor (LIF) was found in the supernatant of 14-day-old cultures, while IFN made early in infection (3–20 days) has also been associated with resistance of certain mouse strains to HSV-1 infection. Although cultures of HIV-1 stimulated mouse T cells did not produce IFN when treated with infectious HSV-1, whereas IFN was produced by bone marrow derived macrophage cultures. In humans, the risk of recurrent herpetic lesions was correlated with abnormal lymphokine production and involved a 8.2 kD suppressor factor made by suppressor T cells that appears to block binding of IFN or IL-2 to their target cells [32].

No studies on phagocytosis modified by mediators released from virus infected lymphocytes have been carried out yet. In recent experiments, peripheral blood lymphocytes were infected by Ads or HSV-1 as well as activated by phytohaemagglutinin (PHA). Their supernatants containing no infectious virus were mixed to phagocytizing peripheral PMNL and MNPh. Beside assaying alterations in

their phagocytic capacity, mediators were partially characterized for their effects on bacterial and eukaryotic cell growth and physical properties.

Materials and methods

Treatment of lymphocytes with viruses and PHA. HSV-1 (strain L2 obtained from Dr. I.F. Barinsky, Ivanovsky Institute, Moscow, Russia), human Ad type 5 (Ad-5, from Dr. H. G. Pereira, National Institute for Medical Research, London, UK), type 8 (Ad-8, strain K isolated from a patient with keratoconjunctivitis and characterized with WHO sera by Dr. A. Lengyel, Institute of Microbiology, Budapest, Hungary) type 12 (Ad-12, strain Huie from Dr. G. Berencsi, National Institute of Hygiene, Budapest, Hungary) were grown in HEp-2 cultures by a standard procedure [45]. Cells detached off culture flasks were centrifuged in a Janetzky K23 centrifuge at 2000 rpm and 4 °C for 10 min, then Ad-infected cells were frozen in dry ice mixed with methanol at -20 °C and thawed in a 37 °C waterbath 6 times. Resulting suspensions were centrifuged again but at 5000 rpm. The latter supernatants were used as partially purified (P) virus stocks, while supernatants after the first centrifugation served as non-purified (NP) stocks. Only purified supernatants of HSV-1 infected cells were used. All of them were stored below -70 °C in small aliquots and 1 tissue culture infectious dose (1 TCID₅₀) as a minimal infectious unit was determined [3].

Lymphocytes were obtained from blood donors not carrying viruses of these types in their lymphocytes as it had been established by indirect immunofluorescence of viral antigens [45] and cocultivating them on HEp-2 and other cultures [45, 46]. Peripheral blood lymphocytes were separated by Halpern's method [47]. Briefly, approximately 400 ml blood was filtered through sterile glass wool at 4 °C, and after 2 h sedimentation, the upper layer containing the majority of T lymphocytes was removed. The cells were washed three times and their number was adjusted to 2.5×10^6 /ml using serum free RPMI-1640 (Phylaxia, Budapest, Hungary) containing 50 U/ml penicillin and 50 µg/ml streptomycin. The viability of cells as determined by the trypan blue dye exclusion test [48] was over 98%. Three ml lymphocyte suspensions were aliquoted in test tubes and infected by 0.1 ml virus stocks containing 1×10^4 TCID₅₀/ml. Another lymphocyte cultures were treated with 0.1 ml phytohaemagglutinin (Difco PHA-P, Detroit, MI, USA) solution at 66 µg/ml, while control cultures were treated with 0.1 ml RPMI only. After incubation at 37 °C for 7 days the supernatants were clarified by centrifugation at 2000 rpm and 4 °C for 15 min. Cell pellets were processed occasionally for electron microscopy, as the method has recently been described [46]. Residual infectious viruses in the supernatants were inactivated by UV irradiation using a Hanau germicide lamp: supernatants were irradiated with approximately 20 erg/mm²/s for 60 min [43]. No infectious virus remained in the supernatants as checked by titration on HEp-2 cultures. One part of each supernatant was dialysed in 4 × 500 ml buffer containing 20 mM TRIS-HCl pH 8.1, 1.75 mM dithiothreitol, 0.025% dimethylsulphoxide (all from Serva, Heidelberg, Germany) at 4 °C for 4 × 30 min. One part of each dialysed supernatant was freeze-dried and reconstituted with RPMI-1640 at desired concentrations immediately before use. Supernatants were stored in 1 ml aliquots at -20 °C until use.

Phagocyte assay. Phagocytes were obtained from the buffy coat of the blood of healthy donors. After three washes in RPMI-1640 the number of cells – a mixture of PMNL and MNPh – were adjusted to 5×10^3 /ml. For the assays, *Staphylococcus aureus* (isolated from a patient and characterized as multiple resistant by Dr. I. Gyarmati, Diagnostic Bacteriology Laboratory, Institute of Microbiology, Semmelweis University Medical School, Budapest, Hungary) was grown in broth at 37 °C for 24 h, subsequently centrifuged in Janetzky K23 centrifuge at 1000 rpm at 4 °C for 10 min. The pellet was resuspended in RPMI-1640 containing 10% calf serum (Phylaxia) and stored at 4 °C, if necessary. Immediately before use the number of bacteria was counted after mixing in 1:1 ratio with standard human red blood cell suspension in smears stained with Giemsa [3] and adjusted to 5×10^4 /ml. For the assay, 250 µl cell suspension was mixed with 250 µl *S. aureus* suspension, the mixtures were completed with 50 µl concentrated or diluted virus-free supernatant of lymphocytes. Other cell-bacterium

mixtures were completed with 50 μ l PHA solution or 50 μ l virus stocks. After incubation at 37 °C for 2 h, mixtures were vigorously shaken, some of the parallel mixtures were washed by centrifugation at 1000 rpm and 4 °C for 15 min three times to remove adhered bacteria. Smears were prepared from the last pellet, stained with Giemsa. Of 50 to 200 PMNL and MNPh both phagocyte index (mean number of bacteria/cells) and the percentage of phagocytes which contain ingested particles, respectively, were calculated [4, 27]. Using the same smears, percentage of dividing staphylococci free and inside phagocytes was established. Photographs were taken in a Zeiss microscope.

All tests were carried out in triplicate, and Student's two-sample *t* test was used for statistical evaluation.

Interleukin and toxicity assays. Interferon (IFN) activity of supernatants was determined using the vesicular stomatitis virus (VSV) yield reduction test [8]. Briefly, for determining total IFN-content, 4×10^4 WISH cells per well in 100 μ l Eagle's minimal essential medium (MEM, Gibco, Paisley, UK) containing 10% fetal calf serum (Gibco), 2 mM glutamine (Gibco), 50 U/ml penicillin and 50 μ g/ml streptomycin were seeded into a 96 well microtiter plate. Next day, supernatants of lymphocytes were clarified by centrifugation in a Janetzky K24 centrifuge at 10 000 rpm and 0 °C for 3 h, then 100 μ l from the middle of clarified supernatants and their twofold dilutions were added to the cell cultures in 10 parallel samples and incubated at 37 °C, in 5% CO₂ for 4 h. Subsequently, these mixtures were replaced by 1×10^2 TCID₅₀/ml of VSV Indiana serotype in 100 μ l Eagle's MEM containing 2% FCS and the usual antibiotics at 37 °C for 24 h (VSV had been grown in WISH cultures, stored at -80 °C, titered at 10^6 TCID₅₀/ml). The IFN- γ and IFN- α content of lymphocyte supernatants – the latter being due to possible contamination of lymphocytes by leukocytes – was established similarly: both lymphocyte supernatants and standards of human IFN- γ and IFN- α preparations (batch CK-9 from the Gamaleya Institute, Moscow, Russia) were incubated in 1:1 ratio with high purity anti-IFN- γ and anti-IFN- α sheep globulin diluted at 1:2500 (obtained from Dr. V. Lackovic, Institute of Virology, Bratislava, Slovakia) at 37 °C for 15 min, then 200 μ l mixture of each combination was added to the cells. Titres were calculated as units (U) per ml upon 50% infection of parallel cultures [8].

Tumour necrosis factor (TNF) activity in these supernatants was determined similarly to IFNs [49]: 4×10^4 L929 cells in 100 μ l Eagle's MEM were seeded in microwells, and next day their medium was replaced by mixture of 100 μ l medium containing 4% FCS and 100 μ l supernatants and their twofold dilutions, respectively, in 10 parallel samples. Cells were stained with 0.5% crystal violet vital dye 24 h later [43] and toxic titres were calculated as in the case of IFNs.

Plating efficiency alterations due to toxicity of supernatants also were determined by the method already has been published [50]. Briefly, 2×10^3 HEp-2 cells in the mixture of 1 ml Eagle's MEM containing 10% FCS (Phylaxia), 20 mM HEPES buffer (Serva), 50 U/ml penicillin, 50 μ g/ml streptomycin and 6 ml UV irradiated supernatant samples were seeded in 70 mm diameter glass Petri dishes in parallel and incubated at 37 °C in the presence of approximately 5% CO₂ for 48 h, when the cells were stained with Giemsa and the number of their foci counted under a microscope was expressed as focus forming unit (FFU) per cm².

Quantitation and analysis of proteins. Protein concentration of supernatants was determined by Lowry's method [51]. Briefly, samples were diluted 100- or 200-fold with distilled water to 200 μ l, mixed with 2.5 ml copper tartrate/carbonate solution for 10 min. Subsequently, 0.25 ml twofold diluted Folin and Ciocalteu's phenol reagent (Sigma, St. Louis, MO, USA) was added to the samples. Protein content was calculated from the optical density at 500 nm as compared to those of the serial dilution of bovine serum albumin (BSA, Sigma) between 0 and 120 μ g/ml.

Polypeptides of supernatants were analysed by polyacrylamide gel electrophoresis (PAGE), as the method already has been published [52]. Briefly, the protein content of supernatant samples was adjusted to 2 mg/ml with 20 mM TRIS-HCl pH 8.0, then 40 μ l samples after adding 2 μ l boiling mixture (containing 6% SDS and some bromophenol blue in the appropriate buffer) and heating them for 2 min at 100 °C were loaded to 1.4 mm thick, discontinuous SDS-polyacrylamide slab gels. Stacking gel contained 5%, while separating gel contained 12% acrylamide (Serva) crosslinked with 0.17% N,N'-diallyltartardiamide (DATD, Serva). Samples were run at 40 mA, then polypeptide bands were

visualized by Coomassie brilliant blue staining. Gels were dried onto Whatman 3 MM thick filter papers using a photograph drier [53]. Bovine serum albumin (67 kD), trypsin (23.8 kD) (both from Difco) and porcine Zn-insulin (11.5 kD, Sigma) at the same concentrations mentioned above served as molecular weight markers when polypeptide bands of supernatants were determined.

Results

Supernatant of lymphocytes infected by HSV-1, Ad-12 and Ad-5 decreased the ratio of phagocytizing PMNL in a concentration dependent manner. The difference between inducing abilities of non-purified and purified virus stocks seemed to be minimal. The effect of Ad-8 is similar to that of uninfected lymphocytes, while PHA treated lymphocytes emitted a factor that increased the ratio of phagocytizing cells (Fig. 1). Changes in the phagocyte index of PMNL exhibited a similar pattern: supernatants of lymphocytes infected with HSV-1 and non-purified Ad-12 suppressed phagocytosis in the highest extent, but approximately, one third of their phagocytic capacity was always retained if more concentrated supernatants were used (data not shown).

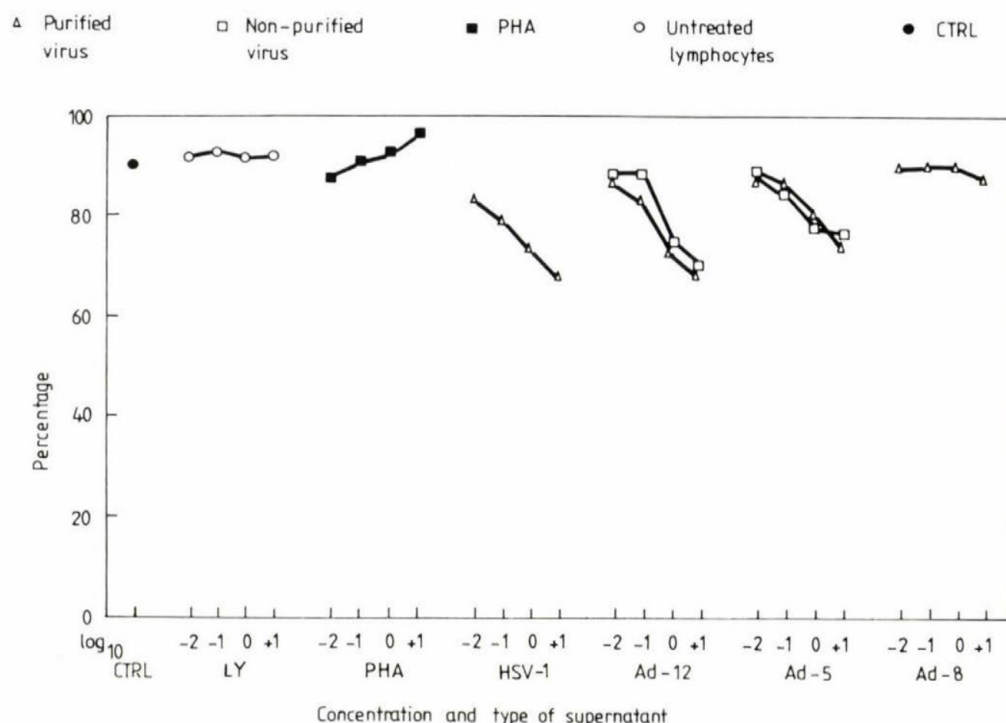


Fig. 1. The effect of supernatants on the ratio of phagocytizing PMN leukocytes (CTRL = control)

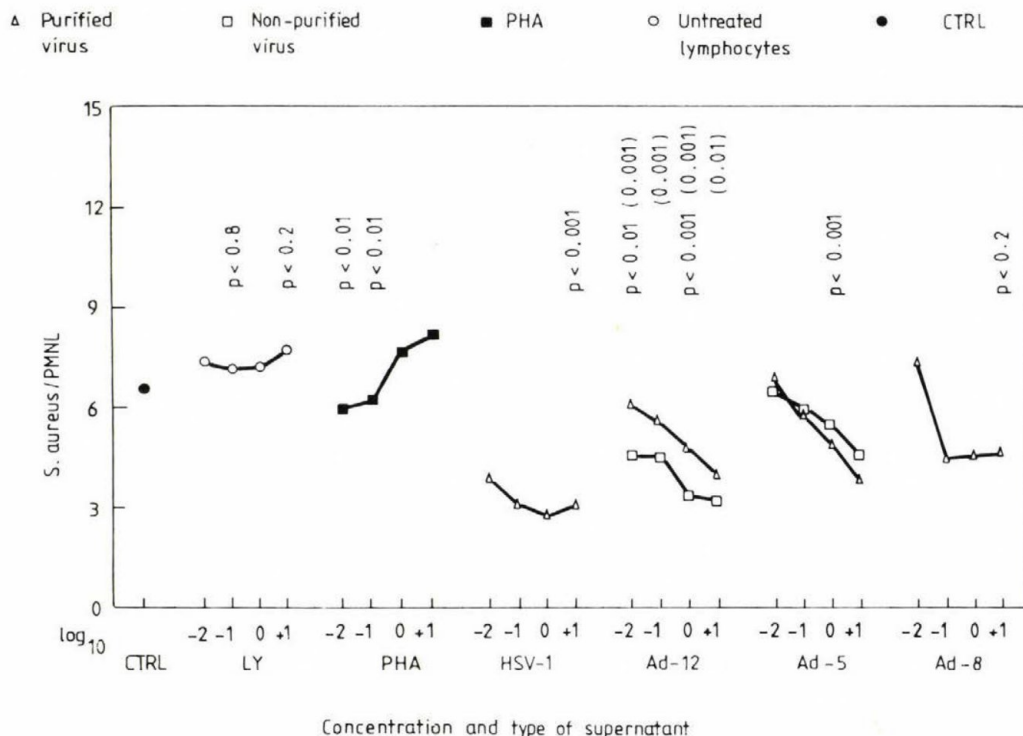


Fig. 2. The effect of lymphocyte supernatants on PMN phagocytosis (P values were compared to the control (CTRL), while P values in brackets represent the effect between purified and non-purified virus stocks, also in subsequent figures)

The difference between inducing capacities of non-purified and purified Ad-12 was significant, while homologous inducing effects were found if two types of Ad-5 preparations were applied (Fig. 2). MNPhs showed extreme sensitivity to decreasing or increasing effects of viral inducers and PHA, respectively. The most pronounced suppression was observed after mixing supernatants of Ad-12 infected lymphocytes (Fig. 3). Viruses added directly to phagocytes also were internalized (Fig. 4). Presence of HSV-1, Ad-12 or Ad-5 affected PMNL activity moderately, while Ad-8 particles and PHA molecules significantly inhibited them. PHA slightly increased the ratio of phagocytizing PMNL, but reduced the average number of ingested microbes. On the contrary, Ad-8 increased the activity of MNPh, while other virus stocks and PHA molecules slightly decreased it (Fig. 5). A two hour period for the phagocyte assay was found enough for staphylococci to divide under ideal conditions of medium and surprisingly inside the cells (Fig. 6).

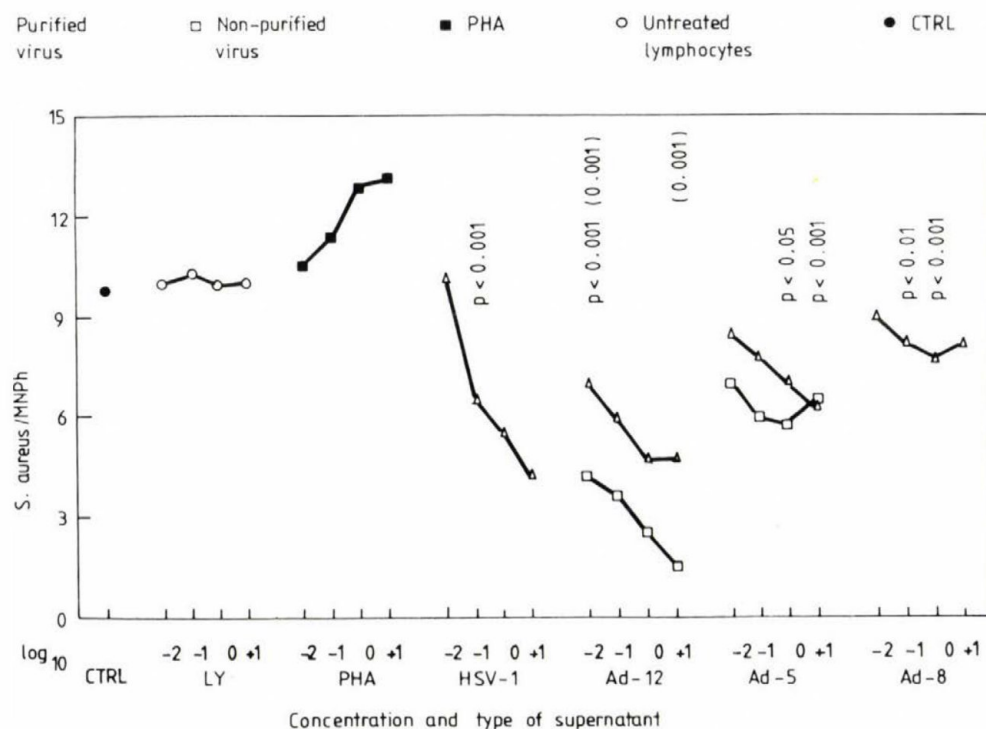


Fig. 3. The effect of supernatants on MNPh phagocytosis

The ratio of dividing bacteria in the medium doubled by the effect of concentrated supernatants of lymphocytes infected by the representative types of the most important Ad groups. Supernatant of PHA treated lymphocytes acted in the same direction (Fig. 7). Inside PMNL, two thirds of microbes were found in diplococcus form, but neither of the lymphocyte supernatant fluids influenced their growth (Fig. 8), while inside MNPhs supernatants of lymphocytes treated with either PHA or Ad-12 increased staphylococcal growth (Fig. 9). The presence of infectious virus particles or PHA molecules hardly influenced the growth rate of bacteria (Fig. 10). Ultracentrifugation of supernatants resulted in the separation of a thick top lipid layer, if lymphocytes had been infected with Ad-12 or HSV-1, while this layer was very thin in the other cases. Supernatants after dialysis and freeze-drying retained their activity.

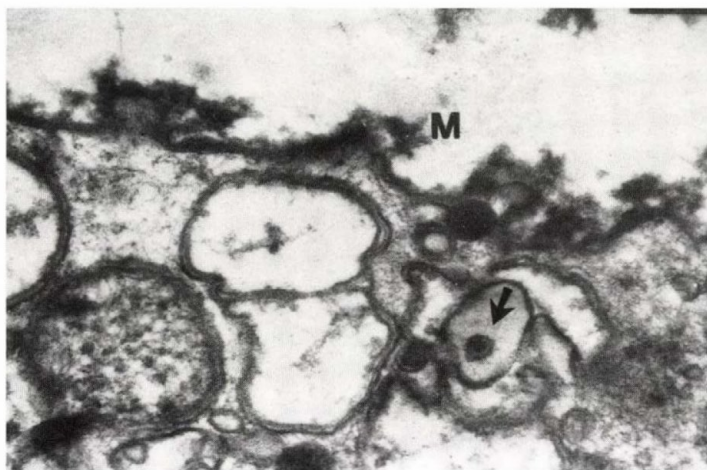


Fig. 4. Electron micrograph on the phagocytic vesicles of PMNL containing HSV-1 particle (arrow) under the cytoplasmic membrane (M), 15 000 $\times$

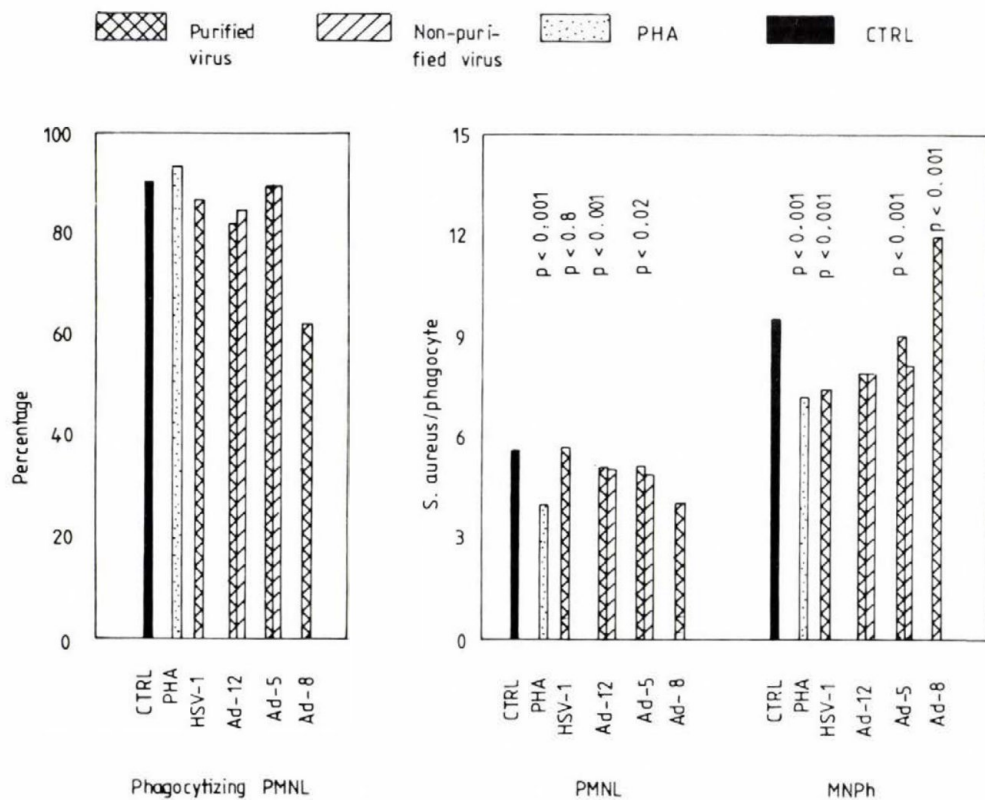


Fig. 5. The direct effect of viruses and PHA on the phagocytosis

Infection with HSV-1 and non-purified stocks of Ad-12 and Ad-5 influenced T lymphocytes to release higher amount of proteins, than purified virus stocks did. Stocks of Ad-8 behaved in the opposite way. By the effect of PHA, protein production of cells was not so high as in the case of control cultures, because of a possible intensive intracellular utilization during blastogenic transformation, while non-stimulated control cultures produced higher amount of proteins again.

Table I

Cytokine and protein contents in the supernatant of treated lymphocytes

Compound	Quantitation	Control	PHA	HSV-1	Ad-12		Ad-5		Ad-8	
					P	NP	P	NP	P	NP
Total IFN	units/ml	32	256	16	64	64	16	32	128	128
Specific activity	units/mg protein	1.8	17.3	0.9	5.4	3.8	1.2	2.3	7.1	8.7
IFN- α	units/ml	0	32	0	0	0	0	0	0	0
Specific activity	units/mg protein		2.2							
IFN- γ	units/ml	32	128	8	64	64	16	32	64	64
Specific activity	units/mg protein	1.8	8.6	0.5	5.4	3.8	1.2	2.3	3.5	4.3
TNF	units/ml	16	4	8	16	8	64	16	16	16
Specific activity	units/mg protein	0.9	0.3	0.5	1.3	0.5	4.9	1.1	0.9	1.1
Total protein	mg/ml	17.8	14.8	16.5	11.8	16.9	12.9	14.1	18.0	14.7

P = purified virus

NP = non-purified virus

In gross, IFN production due to infection with these DNA viruses was found to be very moderate, and the type of IFN was γ , which fact proves the presence of homogeneous T cell population. PHA induced the highest amount of IFN. Supernatants also contained TNF, that reached the highest level, if purified Ad-5 stock was used for induction. In contrast to the highest IFN inducing ability, TNF content was the lowest if lymphocytes were treated with PHA. In our system we could not distinguish between TNF- α and - β (Table I).

Initial analysis of supernatant polypeptides separated by SDS-PAGE showed the presence of 17–19 kD polypeptides in the PHA treated sample. A 50 kD band was observed in the supernatants of Ad-12 infected lymphocytes, and an extra band at 26 kD in the supernatant, if lymphocytes were infected with non-purified Ad-12 stock. Other supernatant fluids did not contain bands in these molecular weight ranges, but all supernatant samples contained several polypeptides above 67 kD (albumin) which are

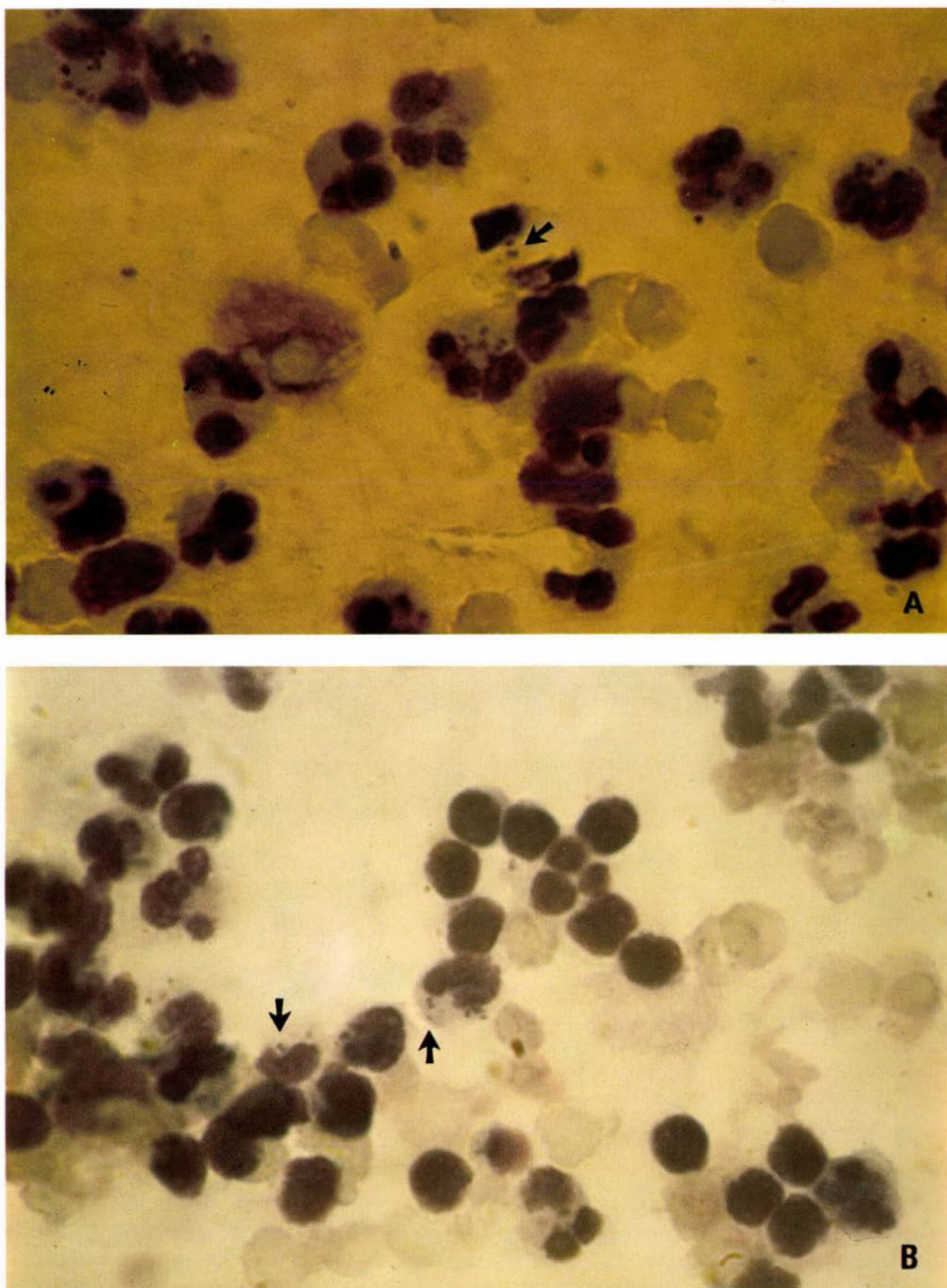


Fig. 6. Phagocytizing PMNL (A) and MNPh (B) containing dividing staphylococci (arrows), 1000 ×

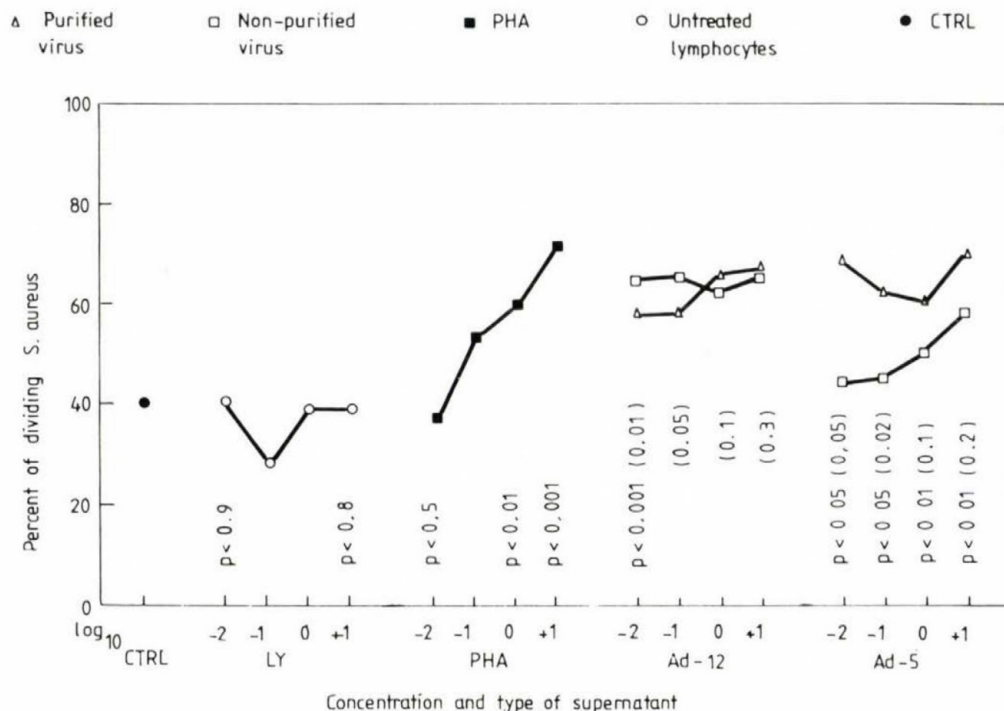


Fig. 7. The effect of supernatants on free bacterial growth

Supernatants of lymphocytes profoundly affected the plating efficiency of HEp-2 cells. Mediators of PHA stimulated lymphocytes had the strongest decreasing effect, while the influence by HSV-1, Ad-12 and Ad-5 was similar and was in the same range. Ad-8 exerted a very mild damage, only (Table II).

Table II

The effect of supernatants of infected lymphocytes on the plating efficiency of HEp-2 cells

Compound	Control	PHA	HSV-1	Ad-12		Ad-5		Ad-8	
				P	NP	P	NP	P	NP
Focus forming units/cm ²	38.5	12.0	24.7	23.4	22.8	21.3	21.9	34.8	32.7

P = purified virus

NP = non-purified virus

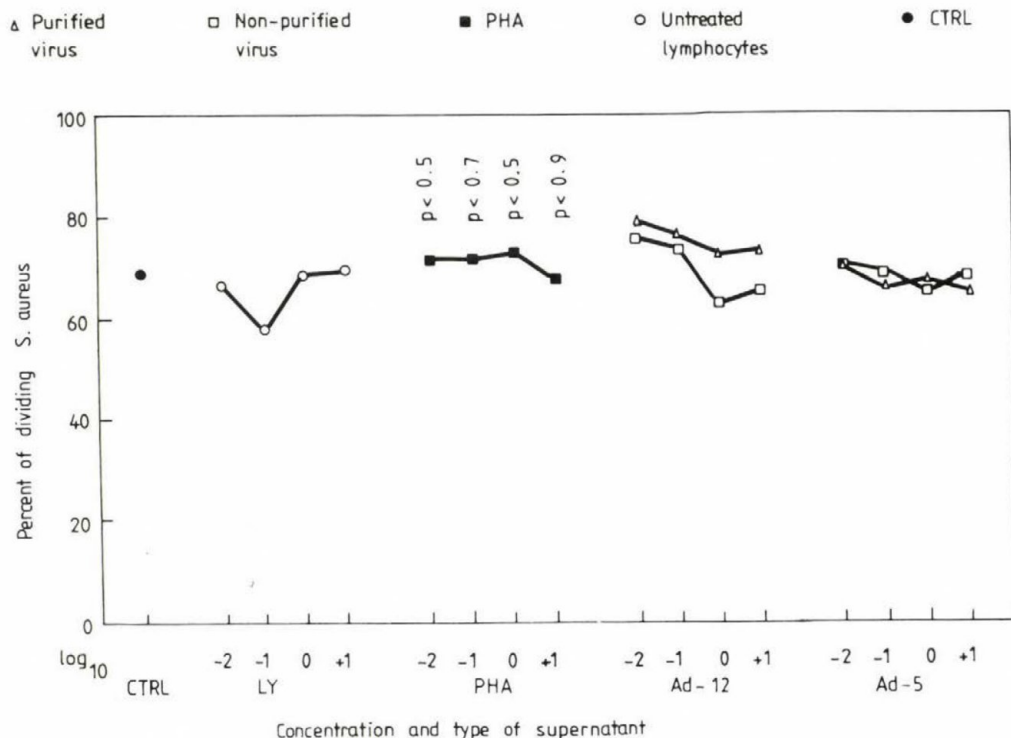


Fig. 8. The effect of supernatants on bacterial growth inside PMNL

The results suggest that the same factor is produced upon infection with different Ads and HSV-1, but effectiveness of induction depends on the viral oncogenic potential. Multiple effects induce at least two independent types of attack on MNPh in contrast to one on PMNL, as well as a direct, but separate effects on human and bacterial cells.

Discussion

Phagocytosis by professional phagocytic cells is important in antimicrobial defence against invading microorganisms [5, 6, 9, 12]. PMNL is the most numerous cell in the human peripheral circulation and the earliest cell to respond to infection, yet its potential in the immune response has been largely ignored [14, 17, 19]. Clinical observations show that infections by several viruses (see references in [3, 4, 9]), among them human herpesvirus 6 (HHV-6), a lymphotropic herpesvirus recognized lately predisposes to neutropenia [11].

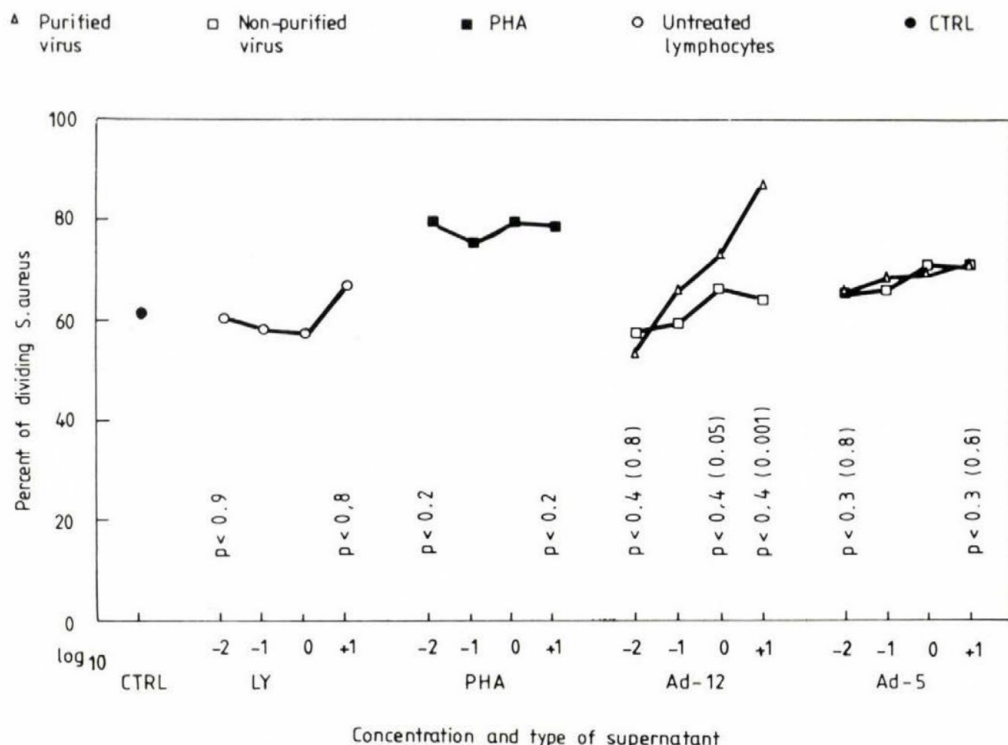


Fig. 9. The effect of supernatants on bacterial growth inside MNPh

Neutropenia can be regarded as a temporary suppression of haematopoiesis known to be directed by several growth factors [10]. Drug misusers infected with HIV-1 are admitted to hospital frequently because of bacterial (*Staphylococcus aureus*, *Haemophilus influenzae*, streptococcal) pneumonia [54]. In an animal model of AIDS, early after infection of susceptible mice with Friend leukaemia virus (FLV), significant decrease in the activity of PMNL was found in spite of that, these cells did not express viral antigens. Restoration of anti-*Candida albicans* activity by simultaneously added LPS suggests involvement of cytokines in modulating PMNL functions [55]. In persons with recurrent herpes infections (labial or genital) a marked disturbance of chemotactic activity and intracellular killing of *C. albicans* was observed in most of the cases. Phagocytic capacity and O_2^- production were normal, but decreased number and impaired transformation of T lymphocytes as well as abnormal macrophage functions were observed [56]. Several types of Ad (types 1, 2, 6 in children, 4, 5, 7, 11 in adults) were found to be associated with *Pneumocystis carinii* or *Mycoplasma pneumoniae* in immunocompromised host [57]. Involvement of several immune functions in upper respiratory tract Ad infection results in neutropenia, while gastrointestinal infections without involving major immune mechanisms do not induce neutropenia [10].

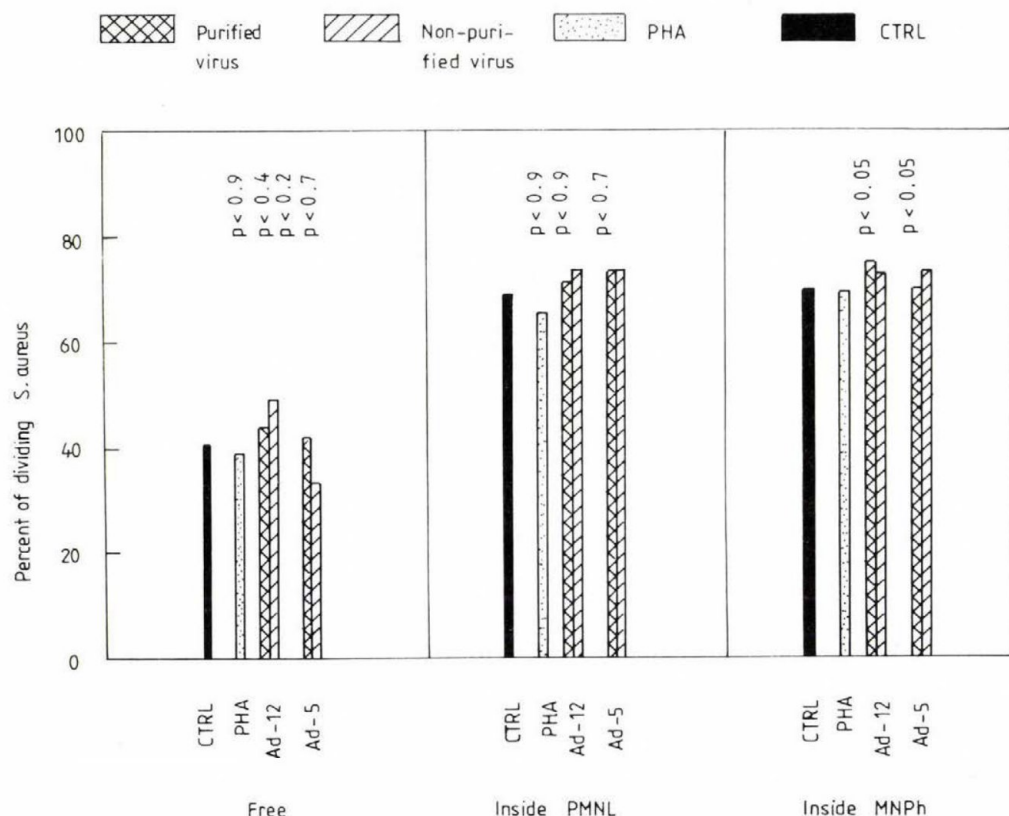


Fig. 10. The direct effect of viruses and PHA on bacterial growth

Ads are regarded as immunosuppressive [29, 35], mainly by expressing multifunctional early (E) genes, which might influence action of cytokines. Although human Ads are lymphotropic and being absorbed, resting lymphocytes are nonpermissive to productive infection [58]. Slight differences exist between human lymphocytes and monocytes in their sensitivity to Ad infection: monocytes genuinely lack receptor for Ad-5, and they continue to grow in its presence. Occasionally, persistent infection is established and a small fraction of monocytes contain multiple copies of free linear Ad DNA [59]. Ad infection would manifest itself with production of infective particles if the organism has already been stimulated by another agent. Infection of an unstimulated cell would result in latent infection or carrier state [31], which phenomenon resembles HIV-1 infection and AIDS [19]. Ad-6 suppressed phagocytosis of red blood cells by mouse peritoneal macrophages, but the virus had no direct toxic effect on macrophages [35]. Ads are found immunosuppressive in mice. It is not known which cells of the immune system are affected [35], because Ad-1 is not

able to replicate in mice [60]. Ad-6 infection in mice depressed NK activity by day 7 as compared to the control group [61], and fluctuations in this and in ADCC reactions [62] of Ad-6 inoculated birds correlated with the IFN production in another experiments [61]. It is well established that Ad-2 or Ad-5 transformed but not Ad-12 transformed rodent cells are relatively susceptible to macrophages, NK cells or cytotoxic T lymphocytes (CTL) [49], because Ad-12 E1A gene product suppresses class I major histocompatibility complex (MHC) [36]. The same Ad product increases low, virus capsid mediated IFN production [41]. Ad-12, but not Ad-5 E1A expression selectively suppresses 27 kD heat shock protein of cells [44]. Ad E3 gp19 kD protein blocks transport of MHC class I antigens to the cell surface [30, 63]. Ad infected cells are rendered sensitive to TNF mediated cytotoxicity by proteins encoded by the E1A transcription unit, but E3 14.7 kD polypeptide protects Ad infected mouse cells from cytotoxicity [30]. Two other proteins, 10.4 kD and 14.5 kD [63], or 11.3 kD and 13.7 kD in other publications [37], functioning in concert can also prevent TNF cytotoxicity. This polypeptide complex has another function, namely, to down-regulate cell surface expression of epidermal growth factor receptor (EGF-R) in Ad infected cells [37, 63]. The larger protein is an integral membrane protein and near to its C terminus it shows significant sequence homology with the juxtamembrane domain at the cytoplasmic face of EGF-R. The expression of the complex attenuates cellular responsiveness to EGF or TGF- α_1 , which latter binds to the same receptor as EGF and is secreted by activated macrophages. In this way the complex might camouflage the infected cells from host responses [37]. E1B 19 kD polypeptide also prevents cytotoxicity of Ad infected human cells, but it does not do this in mouse cells [63]. The expression of the human IFN- β gene is activated by E1B 19 kD protein [41]. Little is known of the molecular biology of Ads in lymphoid cell background. In mouse plasmocytoma cell line MPC-11, a nonpermissive host for Ad viral DNA replication, expression of immunoglobulin genes γ_{2b} and κ was repressed after infection by Ad-5 [39, 64].

Our experiments clearly demonstrate that a component(s) in the supernatant of infected lymphocytes without complete replication cycle of viruses diminishes phagocytosis in a dose dependent manner. Although Ad fiber proteins and penton base were shown to exert toxicity on KB cells inhibiting cellular DNA synthesis and cell clumping, respectively [28], it is not a major route of action on phagocytes, because virions not absorbed decay postinfection gradually [31, 33]. Similarly, Ad-6 had no direct toxic effect on cellular protein synthesis [43]. The exact nature of putative mediators has not been characterized. Their production and effectiveness depended on the virus types used and showed similarities to IFN production [41-43]. Differences between oncogenic Ad-12, HSV-1 and non-oncogenic Ad-5, Ad-8 in the lipid composition of supernatants after centrifugation raises possible involvement of PGs, LTs or another lipid-derived substrates in executing modulatory functions on phagocytes, as it has been shown on dexamethasone treated macrophages [27]. Diacylglycerol components can take part in regulating neutrophil functions [65]. Serum lipid profile is known to be altered by a broad spectrum of infections, which may exert a modifying influence on immunological functions through changes in lipid membrane composition. But it is presumed that alterations in lipid homeostasis could be mediated through cytokines [2, 66]. Effectiveness of mediators in our experiments was retained

for longer period only in frozen state [3], and also after dialysis, which latter refers to their protein nature [2, 26, 27]. Lymphocytes were cultured without serum to avoid interaction with contaminating proteins [2, 26, 33]. Polypeptides in the supernatants therefore solely represented newly synthesized secreted compounds or cellular constituents: 17–19 kD polypeptides shown by SDS-PAGE in the supernatant of PHA treated lymphocytes could represent both 17 kD IFN- γ and TNF- α [19]. Specific activity of interleukins in the supernatant samples indicated the presence of IFN in PHA treated cells, and relatively higher amount of IFN-like substance in the supernatant of Ad-12 and Ad-8 infected lymphocytes in contrary to Ad-5 infected cells. Specific activity of TNF was the lowest in PHA treated samples, while its fluctuation in other samples did not show significant differences. Coordinated production of cytokines is often observed, and this can be modulated by viral infections [23, 25]. Both IFN- γ and TNF- α are known to enhance phagocytosis [14–18, 20, 21] and suppress growth of tumour cells [2, 19, 26, 30, 49], as the same effects were shown in this study by using supernatant of PHA treated lymphocytes. The regulatory effect of these mediators is divergent on normal and tumourous cells, therefore assaying these supernatant samples on several cell cultures would be desirable. 26 kD and 50 kD polypeptide bands in the supernatant of Ad-12 infected lymphocytes might correspond to the 25–26 kD IL-6 and its possible dimer. Several other cytokines, known to affect phagocytes and to modulate eukaryotic cell growth, possess similar physical characteristics: 27 kD IL-5 with 45–50 kD dimers, 20–25 kD glycosylated form of IFN- γ , 25 kD IL-7, 25 kD dimers of TGF- β_1 or 25 kD monomer of TNF- β , etc. [23, 26]. As cytokines exert their effects in pM concentrations [19], initial characterization of their small quantity by Coomassie staining could be followed by silver staining, autoradiography. Western blotting, neutralization with antibodies, etc. [15, 26, 52].

So far less attention has been paid to microbes themselves in phagocyte assays. Staphylococci used frequently in in vitro assays are usually adequately opsonized within 5 min in 5% serum [5], but this process might vary with different strains [67]. Opsonization enhances phagocytosis by binding Fc portion of IgG-opsonized microbes [68] to Fc receptors found among more 40 different types of receptors on phagocytes [8]. Deficiencies in the opsonic activity of serum, such as hypogammaglobulinaemia or complement disorders have been associated with an increased susceptibility to infection [5]. In HIV-1 infected AIDS patients therefore receptor mediated phagocytosis has been found lower [69], while exposure to HSV-1 does not appear to alter the percentage of Fc receptor bearing macrophages, and it does not affect Fc-mediated phagocytosis [7]. Another problem in phagocyte assays is to distinguish between attachment and actual ingestion of bacteria. Electron microscopic studies and use of lysostaphin, an agent than lyses only attached staphylococci have shown the percentage of attached and uningested bacteria to be not more than 5 to 10% [5, 27, 67]. These microbes can be removed through repeated washes by low speed centrifugation, too [27, 67].

Factors released from PHA treated or Ad infected lymphocytes, which significantly inhibited HEP-2 cell growth, showed an opposite effect on staphylococci. Supernatants of PHA treated lymphocytes exerted the highest enhancement on free bacterial growth, while that of Ad-12 infected lymphocytes increased staphylococcal

replication inside MNPh. It has recently been shown that cytokines (IL-1, IL-2, GM-CSF) can bind to specific receptors and enhance growth of *Escherichia coli* and other gram negative bacteria [22, 70] as well as that of *C. albicans* [70]. TNF- α also binds to several Gram-negative bacteria, but has no effect on the growth of *E. coli*. TNF- α coated bacteria had impaired growth in macrophages compared to control treated bacteria [22]. Pretreatment of *Shigella flexneri* had a 20-fold enhancement in cellular invasion [22]. In a recent study we have shown that survival in oral cells after disinfection of Ads and HSV-1 and that of Gram-negative bacteria and fungi are concomitant as compared to the survival of viruses and Gram-positive bacteria [46]. In contrast to Gram-negative bacteria, significantly lower level of TNF- α binding occurred when a number of Gram-positive bacteria, among them *S. aureus*, was assessed [22]. In our recent study, low level TNF in the supernatant of PHA treated lymphocytes corresponds to the promoted growth of free staphylococci. Another study demonstrated that increased serum level of TNF- α in AIDS patients has no role in the secondary microbial infections [72]. Infective dermatitis of children is a recently described HTLV-I associated condition characterized by persistent cutaneous infections, chronic eczema associated with saprophytic staphylococcal or β -haemolytic streptococcal infection of the skin and nasal vestibule. Resistance to treatment, the frequent exacerbations appear to result from a defect in cell-to-cell interactions created by HTLV-I [73].

The events during and immediately after engulfment determine whether the bacterium lives or dies inside the phagocyte. Inhibition of bacterial macromolecule synthesis occurs later, followed ultimately by lysis and digestion by lysosomal enzymes and destroying by ROI, RNI [19]. Neutrophil granules contain a variety of extremely basic proteins that strongly inhibit bacteria, yeast and even viruses. But several pathogenic bacteria have evolved mechanisms to parasitize the mononuclear phagocytes without activating their oxidant defenses or inhibiting the digestive enzymes, therefore are capable of both extracellular and intracellular growth [19]. Staphylococci are not among them. Interestingly, in Chediak-Higashi syndrome, when both phagocytosis and intracellular killing were impaired, in giant phagolysosomes *S. aureus* in active proliferation was commonly observed after 45 min of phagocytic assay [74]. The mechanism of enhanced intracellular replication by staphylococci is not completely understood. These bacteria belong to the most effective ones in triggering and to the most susceptible ones to superoxide anion (O_2^-) and hydroxyl radical ($OH^\cdot$) ions [9]. Diminished production of these extremely short-lived [19] oxygen-derived radicals by the membrane bound NADPH enzyme complex on the effect of mediators, as it has been proven in the case of MDF and TGF- β_1 [21], might let staphylococci and another bacteria [75] survive. Heat shock treatment directly or indirectly through heat shock proteins and activating the membrane-associated oxidase system causes significant enhancement of O_2^- production in macrophages without enhanced intracellular killing of *S. aureus* [9]. The same treatment inhibits O_2^- production in human neutrophils [76], which means that the oxidative system might differ in PMNL and MNPh and external factors can cause a response in MNPh different from that in neutrophils with regard to production of oxygen metabolites [9]. Only MNPh is able to degrade DNA to fragments, because PMNL does not possess DNase [77]. These

facts might explain differences in staphylococcal growth under mediator control inside PMNL and MNPh.

The results presented here pose new questions regarding how interactions between infected cells through soluble mediators might contribute to host defence and/or the pathology of invasive bacterial infections.

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THE IMMUNOMODULATING EFFECT OF A NEW POLYAMINE (THE MAP-1987) ADMINISTERED WITH CHLOROQUINE IN PLASMODIA INFECTED MICE*

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The biological activity of a new synthetic polypeptide, the MAP-1987 was proved in the rodent malaria system. The administration of 4 µg/kg of MAP-1987 prevents the haemolysis of the *Plasmodium berghei* infected erythrocytes but not the *Plasmodium vinckei* infected ones. The MAP-1987 given alone changes neither the survival time of the infected mice nor the rate of parasitaemia. The chloroquine given alone increases the survival time of the mice infected with *P. berghei* under the standardized experimental condition but later the animals die with a low rate of parasitaemia. Chloroquine administered together with MAP-1987 definitely cures the *P. berghei* infected animals. This activity is unique and specific and it does not apply to *P. vinckei* infection.

The polyamines play an important role in the metabolism of the eucaryotic and procaryotic cells. They are ubiquitous in nature, are released from living and dead cells and can be found in physiological fluids. The biology and biochemistry of the polyamines are reviewed in references [1, 2].

Some of the polyamines are considered as immunomodulators, e.g., the ACPS-R recently isolated from the root *Actinidia chinensis* [3] or the PRP, a proline-rich polypeptide isolated from the ovine colostrum [4]. Other polyamines such as spermine and spermidine are associated with non-antibody mediated immune mechanisms to protect against intraerythrocytic stages of malarial and babesial infections [5, 6], the polyamine oxidase (PAO) catalyzing the oxidative deamination of the polyamines [7, 8]. The products are toxic to a variety of cell types, e.g., trypanosomes and intracellular protozoa [9–11]. The characteristic feature of this toxicity is the development of degenerate parasites within the host erythrocytes, the so-called crisis forms described in 1944 first by Taliaferro et al. [12], later by Clark et al. [13]. The role of PAO in the intraerythrocytic killing of *Plasmodium falciparum* has been investigated by Ferrante et

*This paper was written in honour of the memory of Professor Zoltán Alföldy (1904–1992) director emeritus of the Institute of Microbiology Semmelweis University Medical School in Budapest, Hungary

al. [14] and Czepczyk et al. [15]. The activity by the inhibition of monoamine oxidases of exoerythrocytic antimalarials such as primaquine has been described by Brossi et al. [16]. In this paper we give an account of the biological activity of a new synthetic polyamine, the MAP-1987 in the rodent malaria model.

Materials and methods

Reagents. The MAP-1987 was supplied by Biopharm, Budapest. The apparent molecular weight of the polyamine is 500. The MAP-1987 is alcohol soluble and its dilution in water results in a slightly opalescent solution. The biological activity of the solution is stable, the molecular homogeneity is not established. For acute and chronic toxicity the LD₅₀ is 40 mg/kg. Chloroquine phosphate was supplied by the WHO, MAP/RTI.

Parasites and host. *P. berghei* N strain was maintained in CFLP non-Swiss mice weighing 22–25 g, by weekly serial passage using a standard inoculum that produces a uniform disease fatal to 100% of untreated animals with a survival time of 7 days. All animals in any given test were approximately of the same age. The *P. vinckei* and *P. yoelii* strains were maintained as before.

Blood schizontocidal tests were performed according to Peters [17, 18] and Richle [19]. The animals received an intraperitoneal injection of 0.5 ml of heparinized heart's blood with highly parasitized erythrocytes and diluted to contain 2×10^7 /ml parasitized cells. Test compounds were also administered i.p.

In the "single dose test" the compounds were given 72 h after infection of the mice with *P. berghei*, when a 10–15% parasitaemia had developed. In the "four days test" the mice were injected once daily for four consecutive days beginning on the day of infection. In the "D₀+D₁ test" the compounds were administered first 6 h before the infection, then 24 h later. In the "D₀+D₃ test" the compounds were given first 6 h before the infection and this was repeated on the 1st, 2nd, and 3rd days. The parasitaemia was determined daily in the first week, then every two days during the experiment. The survival time of the mice was observed. Each group of experimental animals was composed of 10 mice. For the determination of the haematocrite value the heparinized blood was taken by cardiac puncture before the death of the animals.

Results

Standardization of the inocula. The standardization of the infecting dose of the plasmodia is important in the screening and testing of the antimalarial compounds. The infecting dose is optimal if all mice in the control group die on the same day within a few hours. This critical inoculum and the surviving time is different for each plasmodium species; as the mice were inoculated with the suboptimal doses of *P. berghei*, *P. vinckei* and *P. yoelii* var. *nigeriensis*: with about 10^6 /ml parasitized erythrocytes (Fig. 1).

P. vinckei kills gradually the mice between the 7th and 9th day after the infection with slowly increasing parasitaemia. The survival time of the mice infected with the *P. berghei* and *P. yoelii* became longer and the infection results low parasitaemia (20–25%) and extended mortality until the 11th day.

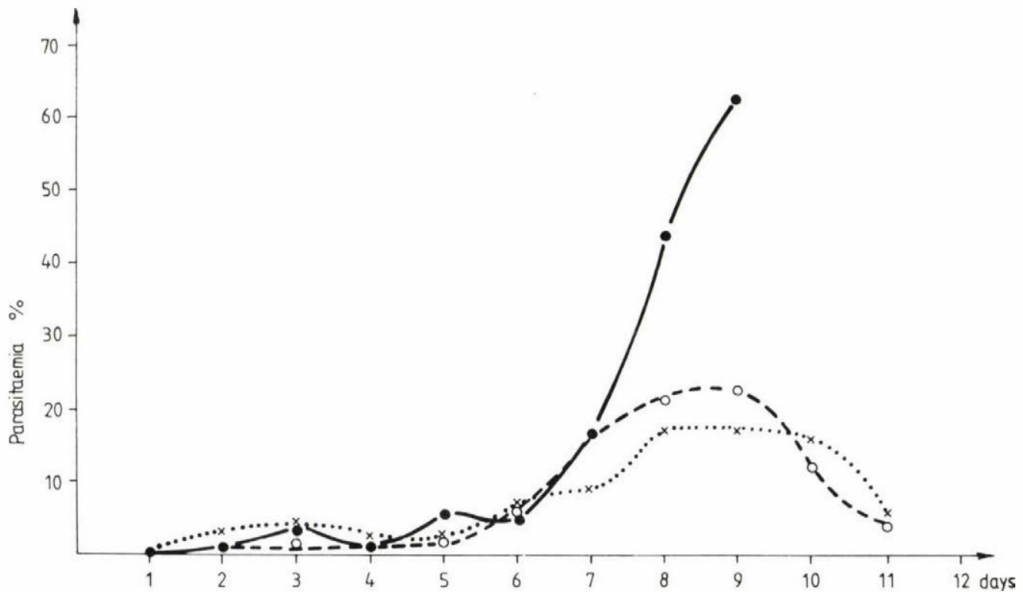


Fig. 1. The course of parasitaemia in mice infected with o --- o *P. berghei* 5.4×10^5 in 0.5 ml; • --- • *P. vinckei* 4.0×10^5 in 0.5 ml; x ···· x *P. yoelii* 4.2×10^5 in 0.5 ml

Increasing the infecting doses of plasmodia the optimal conditions may be reached as it is demonstrated in Fig. 2 for *P. berghei* and in Fig. 3 for *P. vinckei*. *P. berghei* killed the mice on the 7th day with continuously increasing parasitaemia if the inoculum is at least 1×10^7 /ml infected erythrocytes. Decreasing the inocula the survival time became longer and the parasitaemia will be lower. The number of the infecting parasites is not so critical in the case of *P. vinckei* killing the mice regularly on the 6th day but at different levels of parasitaemia.

Selection of the tests. The single dose, the four days, the $D_0 + D_1$ and the $D_0 + D_3$ tests were assessed for selection of the optimal technology. Two groups of ten mice were infected with the standardized quantity of plasmodia and each animal was treated with 30 µg MAP and 1 mg chloroquine, respectively. The parasitaemia was daily measured, the haematocrite value was determined and the survival time was registered. For further experiments the $D_0 + D_3$ test was proved as the most appropriate one and the results presented here are obtained by this technology.

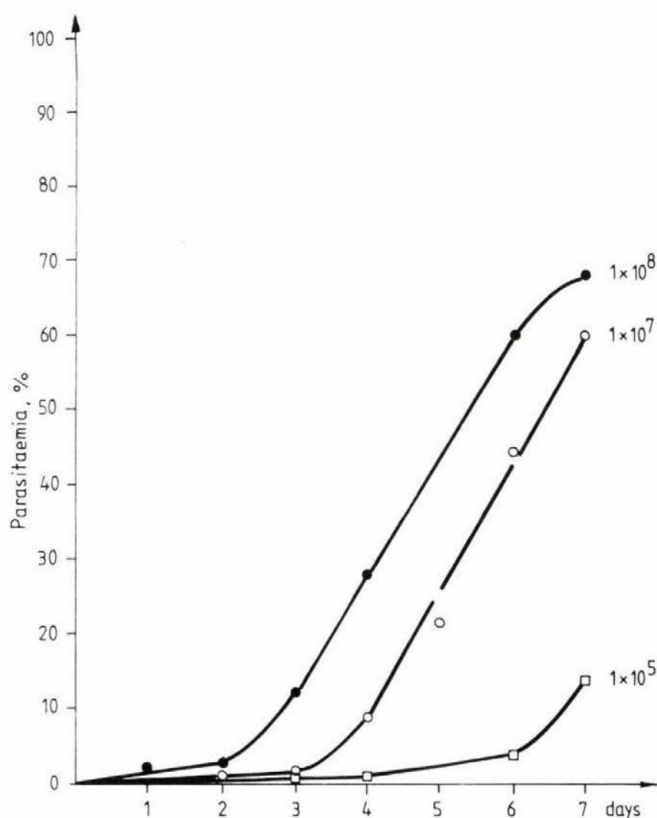


Fig. 2. The course of parasitaemia in mice infected with different number of *P. berghei*

Action of the MAP-1987 alone on the infection with *P. berghei* and *P. vinckei* was investigated and the results are summarized in Fig. 4. Fig. 4/a shows that all of the mice infected with *P. berghei* die on the 7th day after the infection and the survival time of the MAP-treated animals is even shortened by a few hours. The haematocrite value of the non-infected mice (N) is about 45%, in the infected animals (C) about 25% and lysis occurs. In the MAP treated animals haemolysis does not take place and haematocrite value is also normal. Fig. 4/b shows that *P. vinckei* kills the mice on the 6th day, and MAP-treated mice die some hours prior to untreated animals and the MAP does not protect the erythrocytes as it does in *P. berghei* infected animals. The phenomenon may be explained by the fact that *P. berghei* infects mostly the reticulocytes and *P. vinckei* usually the mature erythrocytes. Concerning the activity of the MAP it may be assumed that either the red blood cells or the intraerythrocytic parasites or both are affected.

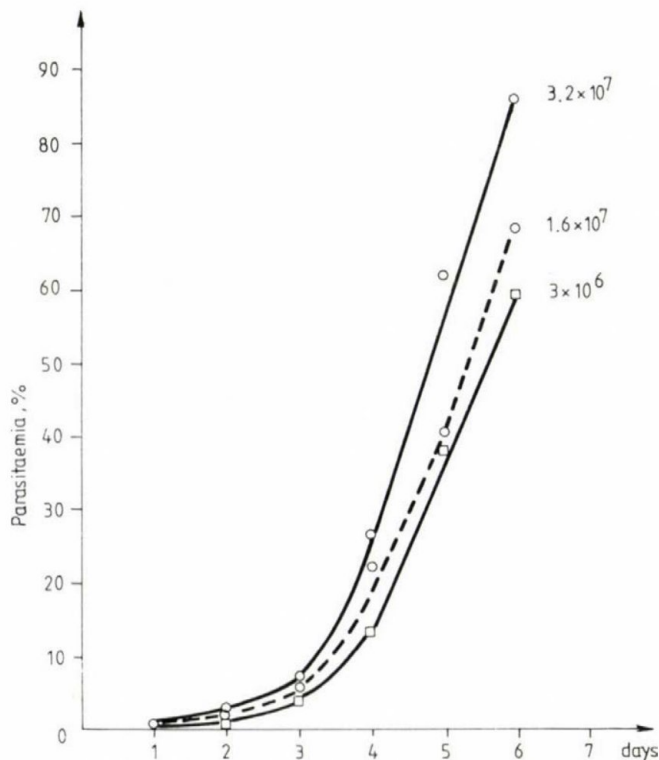


Fig. 3. The course of parasitaemia in mice infected with different number of *P. vinckei*

For proving the working hypothesis, the infected mice were treated with the MAP and chloroquine together in the D₀+D₃ experiment. The results in the case of *P. berghei* infection are summarized in Fig. 5. The survival time was equally 7 days in the control and MAP-treated group of infected animals. The survival time increased to 14 days in the chloroquine treated group. MAP and chloroquine in combination protected the animals and all of them survived the experimental period (34 days).

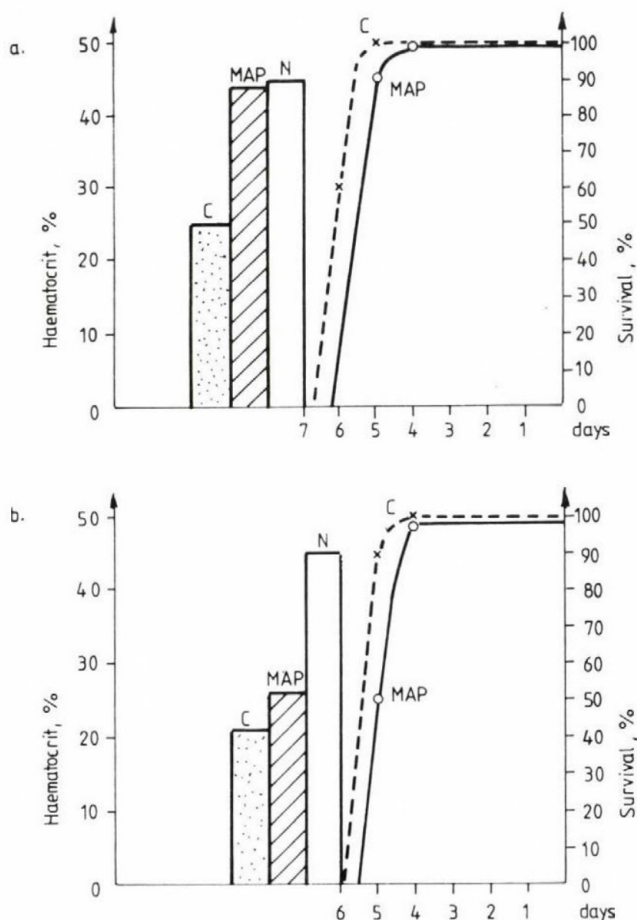


Fig. 4. Activity of MAP-1987 in mice infected with *P. berghei* (a) and *P. vinckei* (b)

In this type of experiment the rate of parasitaemia was also measured in each animals: the mean of percentage value for the group is shown in Fig. 6. The parasitaemia continuously increased up to 70% in the control group before the death of the animals. The MAP-treated mice produced a lower rate of parasitaemia (about 40%), while the chloroquine treated mice died with a low (less than 20%) parasitaemia. The curve for parasitaemia in the MAP and chloroquine treated mice showed a smaller peak on the 15th day and thereafter the parasites disappeared.

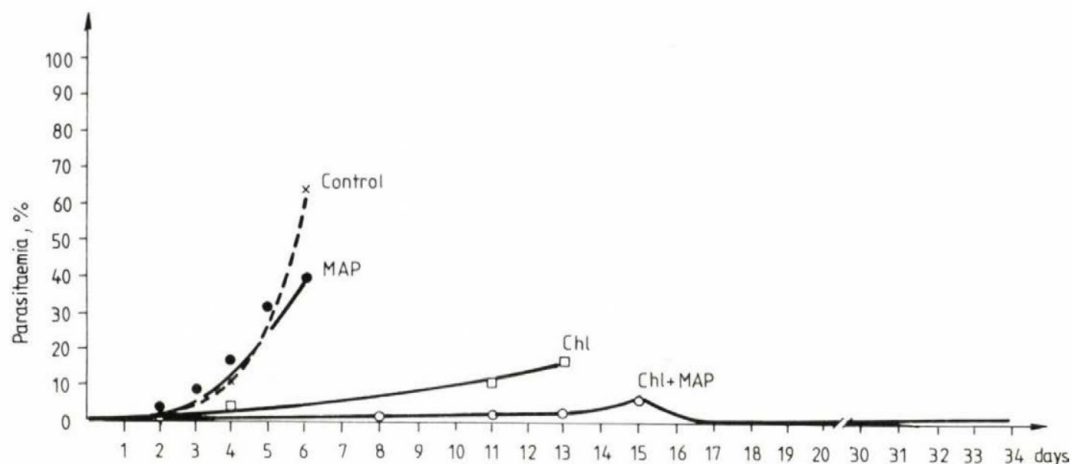


Fig. 5. The course of parasitaemia in the *P. berghei* infected mice in the D_0+D_3 experiment

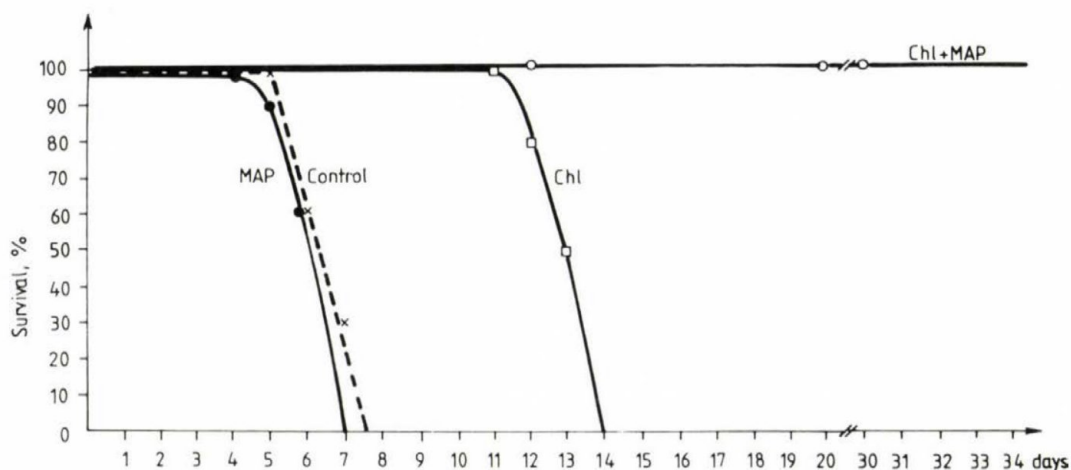


Fig. 6. Survival of the *P. berghei* infected mice in the D_0+D_3 experiment

Interesting are the results of the same type of experiment with the *P. vinckei* infected animals shown in Fig. 7.

Difference was expected on the basis of the haematocrite values. The *P. vinckei* killed both the MAP-treated and untreated mice after the same time. The survival time of the chloroquine treated group decreased to 70%, similarly to the MAP- and chloroquine-treated groups. Figure 8 shows the rate of parasitaemia in the same experiment. The rate of parasitaemia increased continuously up to 90% both in the

control and the MAP-treated groups on the 5th day and there was a peak (30%) in the chloroquine and the MAP plus chloroquine treated groups on the 12–14 days. Thereafter the number of parasites decreased to zero.

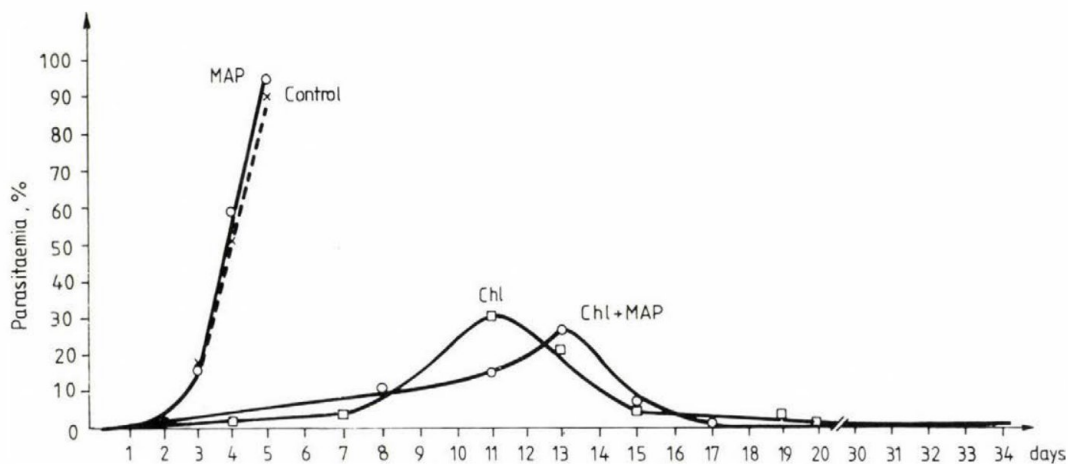


Fig. 7. The course of parasitaemia in the *P. vinckei* infected mice in the D_0+D_3 experiment

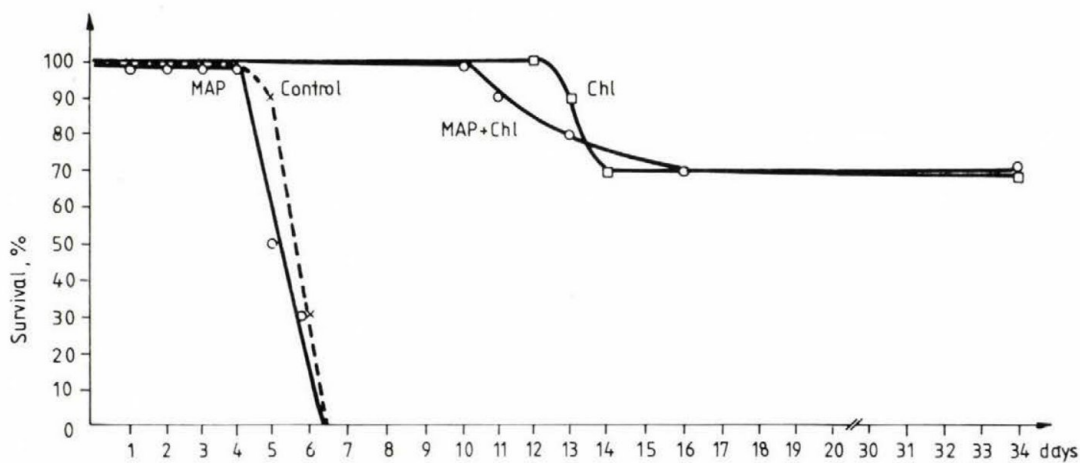


Fig. 8. Survival of the *P. vinckei* infected mice in the D_0+D_3 experiment

The polyamine MAP-1987 is well tolerated by the animals. The acute and chronic toxicity tests proved that the LD₅₀ is much higher than the dose administered in this experiment (30 µg). However, experiments were performed to determine the lowest optimal dose which in combination with chloroquine protect the mice from the lethal *P. berghei* infection. MAP-1987 was administered in gradually decreasing doses up to 0.1 µg (40 µg/kg) to the groups of mice and it was found that even 40 µg/kg prevents the lethal infection.

Discussion

MAP-1987 is a newly synthesized polyamine of biological activity. It mediates some mechanism which kills the intracellular protozoon *P. berghei* if administered together with chloroquine, but leaves *P. vinckei* unaffected. The phenomenon has not yet been characterized. Some data about the non-antibody mediated killing of *P. falciparum* in the erythrocytes [17–19] may help to explain the experimental data. Ferrante et al. [14] and Czepczyk et al. [15], using a number of various isolates of *P. falciparum* cultures, found that the addition of polyamine, spermine, spermidine and PAO to the cultures resulted in degenerate parasites, the crisis forms [13]. But if one or the other were added alone, the cultures were similar to the control. They supposed a direct action of products generated by the oxidation of polyamines on the parasites. The polyamine oxidation results in the formation of toxic aldehydes, hydrogen peroxide via a free radical mechanism and ammonia.

Others found a close correlation between the non-specific tumour killing and the killing of *Plasmodium* and *Babesia* species [20–22]. The bacterial lipopolysaccharides (LPS) can cause also the release of substances from macrophages which are able to kill the intracellular parasites [23]. All of these factors such as the tumour necrosis factor (TNF), the reactive oxygen species, LPS, interferon, PAO, might be the mediators of the non-specific immunity. However, it was proved [15] that ammonia, hydrogen peroxide and associated reactive oxygen intermediates produced during the oxidation of the polyamines were not the cause of the parasite's death in the spermine-PAO system. They considered the aldehydes and breakdown products of these, as effector substances of the PAO mediated killing of *P. falciparum*. Some investigators supposed that the malaria crisis forms are the results of the TNF action [23–26]. This was not proved by Jensen et al. [27] and they stated that neither purified mouse nor recombinant-DNA produced human TNF induced crisis forms in cultured *P. falciparum*. According to Clark et al. [28, 29] the free oxygen radicals generate the lysis of the cells and the hydroxy radical is the most damaging to the cells. In our experiment the MAP-1987 prevents the lysis of the *P. berghei* infected erythrocytes.

The results of our experiments show that the MAP-1987 has a double effect: the prevention of the lysis of the *P. berghei* infected cells and a "synergistic" action with the chloroquine. The above-mentioned facts and hypotheses are not applicable to this unique and specific phenomenon. It is conceivable that the MAP-1987 works in other way(s), either by changing the permeability of the cells or by induction of enzymes leading to the accumulation of the chloroquine in the parasites.

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DETECTION OF TRANSFUSION-ASSOCIATED HEPATITIS CAUSED BY NON-A, NON-B, NON-C FLAVIVIRUS*

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Sera of patients suffering from acute hepatitis, and different forms of chronic hepatitis were found to be reactive to reagents prepared from the yellow fever virus (YF) vaccine strain. Serum samples of 1974 patients were tested, and 133 of them were positive. Hepatitis C virus specific antibodies were absent from the majority of them. The frequency of antibodies to other flaviviruses (tick-borne encephalitis, West Nile) and hepatitis B virus markers was similar to that measured among the population in Hungary positive for any of the surrogate markers of hepatitis infections. Results of both immunofluorescence tests, and Western blots suggest that there is a non-A, non-B, non-C hepatitis virus circulating among the Hungarian population, which possesses antigenic cross-reactivity with the yellow fever virus, but the identity to any of the known flaviviruses could not be verified yet. No history of yellow fever vaccination could be revealed in any of the patients included into this study. The anamnestic data on previous transfusions or surgical operations can be verified only in the case of the half of YFV-positive patients, nevertheless, the sexual transmission seems to be very infrequent. Attempts are continued in order to detect the viral RNA using polymerase chain reaction, and clone c DNA sequences for sequence analysis.

The existence of the endemic (or post-transfusion) non-A, non-B hepatitis has been recognized almost twenty years ago [1]. The most recent discovery of hepatitis C virus [2, 3] proved, however, that it is only one of the aetiological agents causing non-A, non-B hepatitis cases [4–6]. There are series of evidences suggesting the existence of other parenteral non-A, non-B, non-C agents (hepatitis F and beyond) [7, 8]. Phillips et al. [9, 10] described paramyxovirus-like viral particles associated with and possibly the aetiological agent of, syncytial giant-cell hepatitis.

The hepatitis C virus (HCV) was taxonomically specified as a human pestivirus, which is related to the yellow fever virus (YFV), tick-borne encephalitis virus (TBEV)

*This paper was written in honour of the memory of Professor Zoltán Alföldy (1904–1992) director emeritus of the Institute of Microbiology Semmelweis University Medical School in Budapest.

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and the West Nile (WN) virus. While the genetic structure of the hepatitis C and that of the YFV is similar, their nucleotide sequences differ from each other significantly [2, 3, 11, 12].

Cross-reactive antibodies have been identified using dengue virus antigens and HCV first generation ELISA reagents [13].

Antibodies reacting with YFV antigens were recognized in the sera of patients suffering from non-A, non-B hepatitis, and the reacting sera were found to be non-reactive to different HCV reagents [14]. The serological data indicating the existence of an as yet unidentified flavivirus, seemingly able to cause hepatitis and is present in the Hungarian population are described in this paper.

Materials and methods

Preparation of YFV antigens for the detection of cross-reactive antibodies in indirect immunofluorescence tests: Vero cells were infected with the 17D strain of YFV taken from the commercial live tissue culture vaccine Arilvax [15]. The infected cells were incubated at 37 °C for 5 days then the cells were harvested by trypsin and fixed by acetone at room temperature for the use in immunofluorescence (IF) test. The tests have been performed using the indirect IF technique [16].

Serum samples. All sera positive for IgG antibodies to YFV (anti-YFV IgG) were controlled using uninfected Vero cells to exclude the presence of non-specific antibodies. The sera were fractionated using QAE-Sephadex for the detection of specific IgM antibodies [17]. Serum samples of persons vaccinated with live YF vaccine were used as positive controls. The antibodies to hepatitis B core antigen (anti-HBc) and hepatitis B surface antigen (HBsAg) preparations were detected by commercial ELISA kits according to the prescriptions of the manufacturers (ABBOTT Diagnostic Division, Organon Teknika). The antibodies to hepatitis C virus (anti-HCV IgG) were also tested by commercial ELISA reagents (ABBOTT Diagnostic Division, Organon Teknika) verified by Western blot (Chiron RIBA HCV test system second generation assay purchased from ORTHO) [18]. Antibodies to tick-borne encephalitis and West Nile viruses were detected by the indirect immunofluorescence procedure. Antibodies to rubella virus were examined with the haemagglutination inhibition test [17].

In order to exclude non-specific reactions all sera reacting with virus-infected cells were also tested with uninfected tissue culture cells. Only sera without cross-reacting antibodies to cells were considered to be positive to the viral antigens in the reagents.

All sera were tested for the presence of rheuma factor using heat-aggregated human gamma-globulin, and all IgM positivity obtained with rheuma factor positive sera were considered to be indeterminate.

Results

Sera of 1974 hepatitis patients negative for hepatitis A IgM and for HBsAg specific markers, which exclude the possibility of acute HBV infection were examined. Cross-reactive IgG antibodies to YFV antigens could be detected in sera of 133 patients.

Titres of YFV specific antibodies were found to be in the range of 1:40 and 1:80 in sera of both persons vaccinated with YF vaccine and patients with hepatitis.

The sera containing IgG were examined for the presence of IgM. IgM antibodies reacting with YFV antigens were found in the sera of 19 persons.

The immunofluorescence obtained with the YFV antigen preparation is shown in Fig. 1. The antibodies of a YFV vaccinee (a), and the antibodies of the NANBNC patient (c) gave very similar IF pattern using anti-human gamma-chain specific fluorescein conjugate.

Because of the serological cross-reactivity of HCV to dengue viruses [13], cross-reactive antibodies were supposed to be present in the sera positive for YFV antibodies. Therefore, the sera were tested for antibodies cross-reacting with other flaviviruses (i.e. with tick-borne encephalitis, West Nile, as well as rubella virus-infected cells) using indirect immunofluorescence tests.

Table I

Tests of yellow fever virus cross-reacting sera with other antigens

Tests with	Number of sera tested	Number of positive sera
Hepatitis C virus	54	13
Tick-borne encephalitis virus	20	1
West Nile virus	20	0
HBsAg	44	1
anti-HBc	42	10

The results of the comparative experiments are shown in Table I. All sera tested with the different antigens were positive for YFV-reactive IgG, Seropositivity for HCV antigens was tested by first generation ELISA reagents.

Thirteen of 54 sera proved to be positive for HCV, too. Twenty sera were tested with both TBE virus and WN virus reagents, and only one single sample was positive for TBE virus related antibodies. The lower part of Table I shows seropositivity of YFV-positive sera tested with ELISA reagents detecting HBsAg (1 positive out of 44 sera), and anti-HBc (10 positives out of 42 sera).

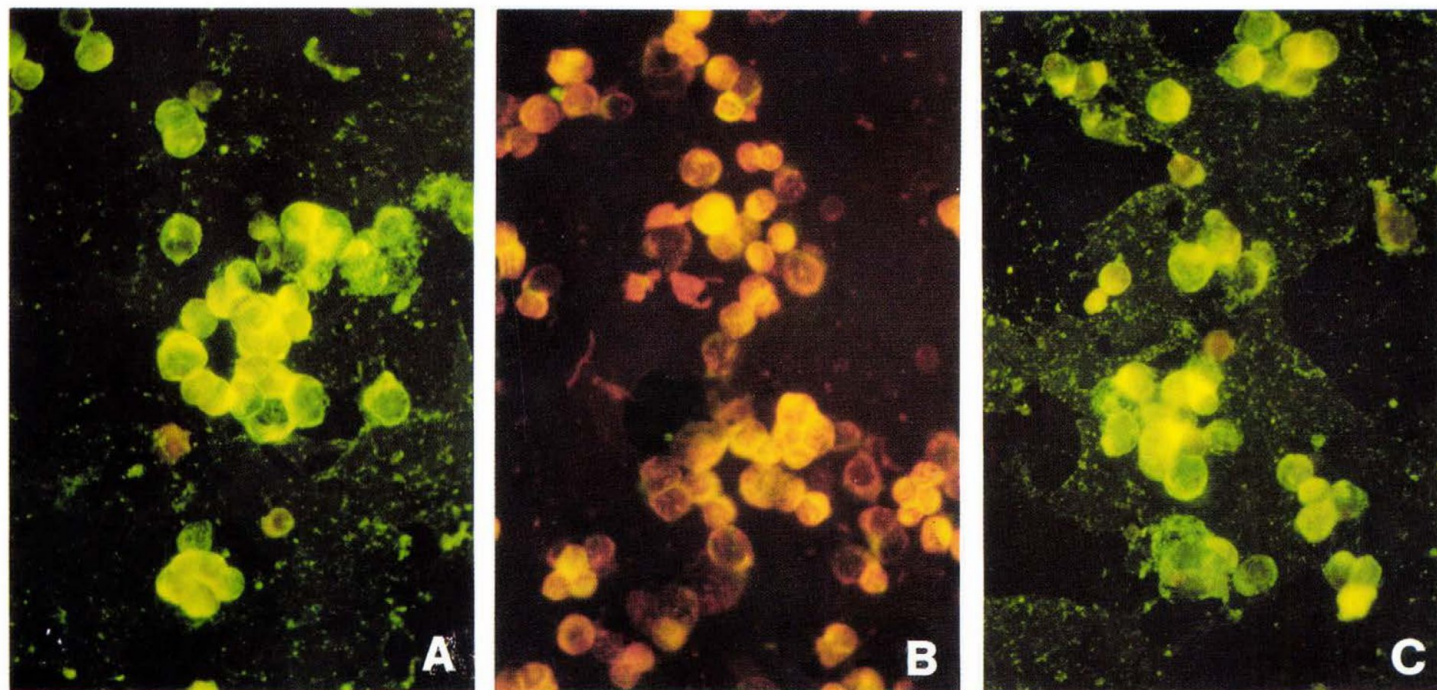


Fig. 1. The appearance of indirect immunofluorescence of yellow fever virus-infected Vero cells detected with anti-human gamma-chain specific conjugate. (A) reaction with the serum sample of a vaccinee (1:10 dilution, vaccination date: 18 months before the sampling). (B) blood sample No. 4998/91, non-reactive serum sample (1:10). (C) blood sample No. 6188/90 (dilution 1:10 taken 12 weeks after the onset of jaundice, female patient of 39 years of age). All samples have been incubated for 150 min at 37 °C with patients' sera, then washed 3 times in PBS, and the 1:60 dilution of DAKO anti-human gamma-chain conjugated with fluorescein isothiocyanate has been used for the detection of IgG antibodies. Photography was performed with an OPTON Axioplan UV Microscope et primary magnification of 400 using indirect illumination [16, 17]

Table II

Tests of seropositive sera for the presence of cross-reacting antibodies with yellow fever virus antigens

Seropositivity with	No. of sera tested	No. of positive sera
Hepatitis C	35	5
Tick-borne encephalitis	20	1
Rubella	10	1
HBsAg	18	1
anti-HBc	28	10

Table III

Comparative examination of serum samples

Sera	anti-YFV	anti-HCV	anti-TBEV	Antibodies to antigens of HCV*				RIBA evaluation (HCV)
	IgG IF	IgG ELISA	IgG IF	a	b	c	d	
1.	pos	neg	n.t.	—	—	—	—	neg
2.	pos	neg	n.t.	—	—	—	—	neg
3.	pos	neg	n.t.	—	—	—	—	neg
4.	pos	neg	n.t.	—	—	—	—	neg
5.	pos	neg	n.t.	—	—	—	—	neg
6.	pos	neg	n.t.	—	—	—	—	neg
7.	pos	neg	n.t.	—	—	—	—	neg
8.	pos	neg	n.t.	—	—	—	—	neg
9.	pos	neg	n.t.	—	—	—	—	neg
10.	pos (vaccinee)	neg	pos (vaccinee)	—	—	—	—	neg
11.	neg	n.t.	pos	—	—	—	—	neg
12.	neg	n.t.	pos	—	—	—	—	neg
13.	neg	n.t.	pos	—	—	—	—	neg
14.	neg	n.t.	pos	—	—	—	—	neg
15.	pos	pos	n.d.	—	—	+	—	indet
16.	pos	pos	n.d.	—	—	+	—	indet
17.	pos	pos	n.t.	—	—	+	+	pos
18.	pos	pos	n.t.	—	—	+	+	pos
19.	pos	pos	n.t.	—	+	+	—	pos
20.	neg	pos	n.t.	+	+	+	+	pos (control)
21.	neg	neg	n.t.	—	—	—	—	neg (control)

n.t. = not tested

* a:5-1-1, b:c100-3, c:c33c, d:c22-3

In Table II serum samples positive for antibodies to HCV, TBEV and rubella virus were tested for the presence of YFV antibodies. The results are similar to those in Table I. The positivity is due to double infection rather than cross-reactivity.

Because of the high number of sera containing antibodies to both YFV and hepatitis C antigens, some of these sera were examined using Western blot (RIBA manufactured by ORTHO). The RIBA contains four HCV specific antigens. The results were summarized in Table III. Nine sera containing anti-YFV IgG but negative for anti-HCV with ELISA were tested by RIBA. All the sera gave negative reaction. The serum of a person vaccinated against YF proved to be negative with RIBA, too. The five sera positive for anti-YFV IgG and a HCV IgG (with ELISA) were either positive [3] or indeterminate [2] with RIBA. All five double-positive sera reacted only with one or two HCV specific antigens present on the RIBA strips. To find possible cross-reactions between the hepatitis C and the tick-borne encephalitis viruses, four sera positive for anti-TBEV IgG were tested with RIBA. All the sera proved to be negative for anti-HCV.

Discussion

The presented results suggest, that an as yet unidentified flavivirus is circulating in the Hungarian population, which cross-reacts with the YFV antigens. It may be responsible for a certain part of the non-A, non-B, non-C hepatitis cases. The examinations have to be continued and extended.

None of a YFV positive patients had been vaccinated against yellow fever, and no one travelled to geographical regions, where the YFV is endemic. Some of the IgM positive patients has undergone surgical operation or blood transfusion one to two months before the onset of their illness. Serum samples could be obtained from the sexual partners of five patients. These sera proved to be negative for YFV antibodies, indicating a low frequency of sexual transmission of this virus.

Some YFV positive sera proved to be positive for HBsAg (1 out of 44) and anti-HBc (10 out of 42) the frequency of anti-HBc positivity is very similar to that of anti-HCV seropositivity (13 out of 54; Table I). The frequency of seropositivity with YFV antigens is even higher (10 out of 28) in the sera of patients who had been recovered from HBV infection (anti-HBc in Table II). These data indicate that the infection with one hepatitis virus is representing higher risk to be infected with other hepatitis viruses, and there is no detectable serological cross-reactivity between the YFV-related virus and HCV antigens present in the first- and second-generation tests used in this study, as well as with the recombinant antigens attached to the immunoblot RIBA strips used in this study.

The sera double-positive for both anti-YFV and anti-HCV were found to react only with one or two HCV specific antigens of RIBA strips. This finding might suggest that the double infection reduces the formation of HCV-specific antibodies in doubly infected individuals.

Acknowledgement. The skilled technical assistance of Mrs Cecília Kerékgyártó-Zalka is very much appreciated. This work was supported by the research grant 1-31/991 from the Ministry of Social Welfare, and the grant No. 785/1991 from the Hungarian Scientific Research Fund (OTKA).

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**THE SECOND CONGRESS OF COST ACRIVAL
ANTIPARASITIC CHEMOTHERAPY**

**SECOND ACRIVAL MEETING ON
CHEMOTHERAPY OF TRYPANOSOMATIDAE
(COST 815)**

BUDAPEST, JUNE 3-4, 1993

ABSTRACTS OF PAPERS AND POSTERS

J. BARBE

**The ACRIVAL COST 815 programme:
outcome and prospect**

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According to the WHO reports, about 10% of the world population suffer from parasitic diseases. With respect to this, trypanosomiasis and leishmaniasis are among the six major infections of humans but, these parasites also cause animal infections which are of a great importance from an economical point of view. Therapy – when it exists ! – is often dominated with drugs of moderate efficacy. There is an urgent need for new agents to treat these diseases, the more so as development of vaccines still remains questionable.

Thus, some European Labs decided to cooperate since 1991 under the auspices of European Communities within the framework of the COST Programme ACRIVAL, with a view to prepare and to investigate new chemicals as trypanocidal agents. Until now, compounds selected mainly belong to the acridine series. Synthetic pathways for the preparation of 9-amino, 9-oxo and 9-thio derivatives concerned in the researches under discussion, will be presented. Attention will be focused on tautomeric problems. Results obtained “in vitro” with these drugs against various trypanosoma and leishmania strains, will be summarized. Preliminary results of “in vivo” investigations in infected mice will be mentioned. A general survey of the possible mechanism of action will be discussed with reference to the intercalating properties which are commonly admitted for acridines. Finally, the development expected in the future will be detailed.

F. A. S. KUZOE

Human African trypanosomiasis

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Switzerland*

Human African trypanosomiasis (sleeping sickness) is a parasitic disease which is caused by infection with trypanosomes, *Trypanosoma brucei gambiense* and *T.*

brucei rhodesiense, transmitted by the tsetse fly *Glossina*. It is fatal, if untreated, and occurs in 36 African countries, south of the Sahara, where some 50 million people are at risk of acquiring infection. At the local level, it is an important public health and socio-economic problem which carries a continuous threat of severe epidemics, which are difficult and costly to control. 15 000 to 20 000 new cases of trypanosomiasis are reported annually but a true estimate of infected people is more likely in the range of 200 000 – 300 000 per year.

The principle of control and prevention of sleeping sickness relies on an integrated strategy of continuous surveillance involving diagnosis and treatment of the population at risk and vector control, where applicable. Thus, chemotherapy is only one but essential aspect of control. Until recently, chemotherapy of African trypanosomiasis relied essentially on three drugs. Pentamidine, a diamidine introduced in 1937, is currently available as pentamidine iso-thionate and is effective against early stage of the *T. brucei gambiense* infection. Suramin, which was introduced in 1922, is effective against early stages of both *T. brucei gambiense* and *T. brucei rhodesiense*. However, its use has been confined to *T. brucei rhodesiense* infections. Melarsoprol, a trivalent arsenical, was introduced in 1949 and until 1990, had been the only drug available for the treatment of late stage *T. brucei gambiense* and *T. brucei rhodesiense* infections. All these drugs have adverse side effects, melarsoprol causing reactive encephalopathy in 5-10% of patients treated, with a fatal outcome in 1-5%. Resistance to pentamidine of *T. brucei gambiense* and melarsoprol of both *T. brucei gambiense* and *T. brucei rhodesiense* has been reported.

Eflornithine (Ornidyl®), a potent inhibitor of polyamine synthesis was registered for the treatment of African trypanosomiasis in 1990 but has many drawbacks which limit its use on a large scale. While it provides the best alternative therapy to melarsoprol for gambiense sleeping sickness, it is ineffective alone against *T. brucei rhodesiense* infection. Besides a complicated mode of administration, it is expensive, the cost of the drug alone is US\$ 210 and the total cost of treatment per patient amounts to almost US\$ 500. Eflornithine is currently not available commercially. Marion Merrell Dow, the manufacturer, has offered the World Health Organization the rights, the patent and technical know how for the production on a royalty free basis by a third party. However, to get eflornithine to countries where it is needed, the immediate task is to find voluntary donors who would contribute to a fund of US\$ 200 000 required to guarantee production of the first batch of the drug. Nevertheless, research is continuing, using modern tools of molecular biology, aiming at developing safe, effective, and inexpensive trypanocides, but the prospect of any new drug for human African trypanosomiasis being produced commercially in the near future is dim, unless the drug would have a potential use against parasites causing diseases, which can sustain the commercial interest of the pharmaceutical industry.

P. KAGERUKRA, J. M. KAZADI and P. VAN DEN BOSSCHE

Trypanocidal effect of Ridzol-S (MSD) to the trypanosomiasis caused by *Trypanosoma congolense*, *Trypanosoma brucei* and *Trypanosoma evansi*

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In spite of the fact, that the mechanism of ronidazol effect has been studied for a long time, the precise details of the trypanocidal activity of Ridzol-S (MSD) remained unclarified. During recent years, the therapeutic experiments have been performed using the oral route of administration. The drug was added to the drinking water or to dry foods (in granular or pelleted forms) of rats or rabbits. The duration of the treatment were between 3 to 9 subsequent days. The concentrate of Ridzol-S was 2 to 20 g/litre in drinking water, or 7300 ppm Ridzol-S in the granular preparations. In case rats have been chosen for the experiments trypanocidal effect was observed in the case of all three trypanosoma species. When rabbits were infected with *T. evansi* the results proved to be incongruent. In combination with potentiating drugs, or other trypanocidal substances the ronidazol was found to be more effective.

P.-H. CLAUSEN, I. SIDIBE, I. KABORE and B. BAUER

Development of multiple drug resistance of *Trypanosoma congolense* in Zebu cattle under high natural tsetse-fly challenge in the pastoral zone of Samorogouan, Burkina Faso

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Preliminary data from an on going epidemiological survey in the pastoral zone of Samorogoua (Kéné Dougou) indicate the occurrence of multiple-drug-resistant *Trypanosoma congolense*. In spite of frequent trypanocidal drug treatments with diminazene aceturate (Berenil[®], Hoechst A.G.) at 7 mg/kg body weight (bw) at intervals of 2 to 4 weeks, no significant drop in the prevalence of African animal trypanosomiasis (AAT) was observed. To examine a suspected drug resistance, 20 Zebu cattle, naturally infected with *T. congolense* and/or *T. vivax*, were transferred in December, 1989 from Samorogouan into a fly-proof stable. Diminazene aceturate at 7 mg/kg bw cured infections of *T. vivax*, but was ineffective against *T. congolense*. Likewise, treatments with homidium bromide (Ethidium[®], FBC Ltd) at 1 mg/kg bw and isometamidium chloride (Trypanidium[®], Rhône Mérieux) at 1 mg/kg bw, respectively, proved to be ineffective. Corresponding chemotherapeutic trials in previously unexposed Zebu bulls and Sahelian goats infected with one primary *T. congolense* isolate from Samorogouan demonstrated a high level of resistance to

diminazene aceturate (7 mg/kg bw in cattle and 17.5 mg/kg bw in goats), isometamidium chloride (1 and 2 mg/kg bw i.v. in goats) and quinapyramine sulphate (Trypacide "S", Rhône Mérieux) at 5 mg/kg bw in goats. The appearance of a multiple drug resistant strain of *T. congolense* emphasizes the urgent need for new chemical substances as trypanocidal drugs and the increasing importance of efficient vector control.

M. BAKHIET, T. OLSSON, B. HÖJEBERG, Å. LJUNGDAHL, C. EDLUND,
G. ANDERSSON, H.-P. EKRE, WAI PING FUNG LEUNG, TAK MAK,
H. WIGZELL, U. FISZER, and K. KRISTENSSON

**CD8 is critically involved in lymphocyte activation
by a *Trypanosoma brucei brucei* released molecule**

Karolinska Institute, Department of Neurology, Huddinge Hospital, Stockholm, Sweden

Trypanosoma brucei brucei released lymphocyte triggering factor (TLTF), which triggered purified CD8⁺, but not CD4⁺, T cells to interferon-gamma (IFN- γ) mRNA expression and secretion, and to ³H-thymidine incorporation. TLTF also induced mRNA for TGF β , but not for IL-4. The action of this TLTF on mononuclear cell (MNC) cultures, was blocked by anti-CD8 antibodies and by soluble CD8. MNC from a mutant mouse strain lacking CD8 expression were not triggered by TLTF. IFN- γ provides a growth stimulus for *T. brucei brucei* and infected CD8⁻ mice had much lower parasitaemia and survived longer than CD8⁺ mice. The host-parasite interaction in experimental African trypanosomiasis thus involves parasite release of TLTF, which by binding to CD8 triggers CD8⁺ cells to produce the parasite growth promoting cytokine IFN- γ .

W. OBEXER, G. SCHMID and R. BRUN

Effect of acridine derivatives on pathogenic trypanosomes

Swiss Tropical Institute, Basel, Switzerland

Eighty-two newly synthesized acridine derivatives of different families were screened as potential trypanocides. They showed a dose dependent effect on *Trypanosoma rhodesiense* and *Trypanosoma brucei* bloodstream forms measured by the inhibition of esterase activity in a fluorescence based in vitro assay. After analysis of the IC₅₀ and MIC values of the investigated acridines it was obvious that no new compound reached the level of the trypanocidal drugs in use (50 μ g/ml). Most of the derivatives had IC₅₀ values in the range of 1 to 10 μ g/ml. Eleven derivatives from

various groups of acridines were in vitro active at 1 µg/ml or below. By comparing the IC₅₀ and MIC values of these compounds, a few correlations between structure and effect on trypanosomes became obvious, whereas no significant differences in the drug susceptibility between *T. brucei* and *T. rhodesiense* was seen. The dialkyl-amino-alkyl derivatives among the group of the 9-thioacridines are more potent than the mono alkylated ones. Tetrahydrothioacridines show, after the hydration in position 1, 2, 3 and 4 the influence of higher substituted side chains on the trypanocidal activity in the same way as 9-thioacridines. Dimers of 9-thioacridines, which are linked covalently by diamino-diphenyl, loose their activity and are hardly soluble in polar solvents. The corresponding ketones of 9-thioacridines confirm the mentioned tendency of increasing toxicity due to the derivatisation of the dialkyl-amino-alkyl side chain. The diisopropyl-amino-ethyl derivative of 9-acridone causes a 50% inhibition of esterase activity at the low level of 0.1 µg/ml in vitro. Within the series of the 9-aminoacridines the elongation of the side chain does not markedly change the activity, however, the IC₅₀ values are generally low between 0.15 and 0.65 µg/ml.

R. KAMINSKY, C. SCHMID, R. NÜESCH and R. BRUN

In vitro cytotoxicity testing of antitrypanosomal compounds

Swiss Tropical Institute, Basel, Switzerland

Screening of compounds for antitrypanosomal activity can effectively be performed in vitro. However, identification of lead compounds for further evaluation does not depend solely on their activity on parasitic trypanosomes. In addition, compounds must not exhibit harmful effects on mammalian host cells at trypanocidal concentrations. Therefore, three different in vitro cytotoxicity assays were investigated for their suitability to obtain early data on acute toxicity of compounds. Subsequent to incubation of various human and animal cells with serial dilutions of compounds (a) changes of cell morphology were determined microscopically, (b) viability of cells was determined with a fluorescence assay, and (c) growth of trypanosomes was quantified with a colorimetric assay. The latter one in which the endpoint is determined by staining of fixed trypanosome-protein with sulphorhodamin B proved to be most suitable. The difference in acute cytotoxicity of compounds on trypanosomes and on mammalian cells was expressed as the "in vitro therapeutic index", which was determined initially for commercially available drugs such as diminazene aceturate (10 000 fold) or melarsoprol (2500 fold).

F. VAN GOOL

**Cymelarsan – a new treatment for *Trypanosoma evansi*
infections in camels: results of field trials in Africa.
Preliminary results in horses and cattle**

Rhône Mérieux, Lyon, France

There are approximately 15 million camels in the world; 12 million in Africa and three million in Near and Middle East countries. Although camels rarely enter the tsetse-belt, and therefore do not usually become infected with the tsetse-transmitted trypanosome species, infections with *Trypanosoma evansi*, which is mechanically transmitted by biting flies, are encountered. Infection rates reach 20% in affected areas and rates as high as 70% have been detected. Treatment of trypanosomiasis in camels was, until very recently, dependent upon two drugs: Suramin and Quinapyramine. Drug resistance against those two drugs, bad tolerance and/or complicated administration, highlighted the need for a novel trypanocidal drug.

For these reasons Rhône Mérieux, France, developed Cymelarsan, a novel trypanocide with very good activity against the trypanozoon sub-genus. As part of the development program the efficacy and safety of Cymelarsan in curing *T. evansi* infections in camels was investigated in different regions of Africa. These field studies in camels (n=88) were performed using both natural infections and artificial challenge, in field conditions and in fly-proof accommodation. Cymelarsan, at a dose rate of 0.25 mg/kg bodyweight, was fully effective against the acute and chronic form of camel trypanosomiasis due to *T. evansi* infections. Cymelarsan eliminated *T. evansi* completely and no recurrence occurred. Preliminary studies in horses and cattle indicate that Cymelarsan could be an interesting trypanocidal drug for the treatment of *T. evansi* in these species.

S. L. CROFT

**Anti-leishmanial and anti-trypanosomal activity
of phospholipid analogues**

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Although several lipids have known antibiotic activity it is only lipopeptides, for example polymyxin and Cerilipin, which have been developed as antimicrobial drugs. During the last decade a group of phospholipid analogues, alkyllysophospholipids, have been developed as anti-cancer drugs. Four of these, ET-18-OCH₃, hexadecylphosphocholine (HDPC), ilmofosine and SRI 62-834, have entered clinical cancer trials.

The anti-leishmanial activities of HDPC (Croft et al., 1987, *Biochem. Pharmacol* 36, 2633–36; Kuhlencord et al., 1992, *Antimicrob Agents Chemother* 36, 1630–34) and ilmofosine (Croft et al., 1993, *Trans R Soc Trop Med Hyg.*, in press) have already been reported. We describe here a comparative study of the activities of ET-18-OCH₃, HDPC, ilmofosine and SRI 62-834 against leishmanias and trypanosomes. In studies on *Leishmania donovani* all four compounds showed activity against amastigotes in macrophages, in vitro, with ED₅₀ values in the range of 1.7 to 7.3 μ M. Against *L. donovani* in BALB/c mice, HDPC and ilmofosine were the most active with ED₅₀ values of 9.2 and 14.5 mg/kg $\times$ 5 (oral), respectively. All four compounds were highly active against *Trypanosoma cruzi* Y strain amastigotes in macrophages in vitro with ED₅₀ values between 0.2 and 1.5 μ M. Ilmofosine, HDPC and ET-18-OCH₃ also had a suppressive effect on *T. cruzi* Y strain in BALB/c mice at 30 mg/kg $\times$ 5 (oral). *Trypanosoma brucei* bloodstream form trypomastigotes, in vitro were also sensitive to all four phospholipid analogues with ED₅₀ values between 7.0 and 43.9 μ M.

The mode of action of these compounds is not clear. The effective concentrations are too low for surfactant activity. Interference with phosphocholine synthesis and incorporation in membranes is possible (Achterberg and Gercken, 1987, *Mol Biochem Parasitol* 26, 277–87). Preliminary studies with ilmofosine and *Leishmania* promastigotes has shown a rapid cytotoxic effect with ultrastructural changes in the cytoplasm and not the plasma membrane.

L. GRADONI, M. GRAMICCIA and M. C. ANGELICI

The genetic polymorphism of *Leishmania infantum*, agent of leishmaniasis in the Mediterranean area

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Until the early 80s, *Leishmania infantum* was considered a genetically monomorphic species, agent of human and canine visceral leishmaniasis. The zymodeme nomenclature adopted for this parasite by Montpellier and London Reference Centres, was MON 1 and LON 49, respectively. The following factors have led thereafter to a drastic revision of the taxonomic status of *L. infantum* in the Mediterranean area: (a) A more accurate isoenzyme characterization; (b) The analysis of endonuclease digestion patterns of the kinetoplast (k) DNA; (c) The successful isolation and characterization of a high number of isolates from Mediterranean cutaneous leishmaniasis cases; and (d) The identification of parasites in an increasing number of HIV-leishmania co-infections.

The current taxonomical status of the *L. infantum* "complex", related to clinical expression, can be summarized as follows: (I) In immunocompetent patients, and in dogs, visceral leishmaniasis is mainly caused by the typical zymodeme MON 1. In some areas, minor genetic variants are present. (II) In immunocompetent patients,

simple cutaneous leishmaniasis is caused by a number of parasite populations genetically distinct from MON 1 ("dermotropic" *L. infantum*), as revealed by both isoenzyme and kDNA analyses. (III) Seriously immunocompromised patients, as those HIV co-infected, display visceral dissemination of parasites belonging to MON 1, dermotropic *L. infantum* and zymodemes which have never been isolated from immunocompetent hosts. These findings strongly suggest that *L. infantum* is a complex of organisms having different virulence to man genetically determined, which may co-circulate in the same endemic area. The clinical outcome in man would represent an equilibrium between this characteristic and the host's immunitary status.

R. BRUN, R. KAMINSKY, J. STANEK and H. METT

**Evaluation of new polyamine synthesis inhibitors
for trypanocidal activity**

*Swiss Tropical Institute, Basel, Switzerland, and Pharmaceuticals Research Laboratories, Ciba-Geigy Ltd,
Basel, Switzerland*

Series of inhibitors of ornithine-decarboxylase (ODC) and of S-adenosylmethionine-decarboxylase (SAMDC) have been tested for their trypanocidal activity against bloodstream forms of *Trypanosoma rhodesiense*. Two in vitro assays were used, both carried out over 72 h in microtiter plates with serial dilutions of the inhibitors. In one assay the minimal inhibitory concentration (MIC) was determined, with the other test method, a fluorescence assay, an IC₅₀-value was determined. Six of the SAMDC inhibitors showed MIC or IC₅₀-values of 1 µg/ml or even below. Four ODC inhibitors, including DFMO, were much less active with MIC-values between 20 and 50 µg/ml. Toxicity for two human cell lines was assessed using two in vitro assays. Minimal inhibitory concentrations for six of the most active inhibitors were around 500 µg/ml. By comparing the MIC values for human cells with those for trypanosomes, an in vitro "therapeutic index" was calculated. Factors over 1000× were found for some of the compounds whereas for DFMO a factor 22× was calculated. The strong trypanocidal activity, the wide therapeutic index of some of the SAMDC inhibitors as well as promising in vivo results in a mouse model encourage further investigations.

J. FÖLDES

The MAP-1987 lipopolyamine as a potential immunomodulant in the protozoon diseases*Department of Clinical Microbiology, Albert Szent-Györgyi University Medical School, Szeged, Hungary*

The MAP-1987 potentiates some chemotherapeutic agents. Its activity was investigated in malaria infected mice during the course of chloroquine treatment. The Swiss mice were infected with *Plasmodium berghei* (N strain) and treated with chloroquine according to the principles of the "single dose" test of Peters as well as to the "four days test" recommended by Richle. The chloroquine (40 mg/kg) and the MAP-1987 (40 µg/kg) were administered alone and combined. The rate of parasitaemia of the infected mice was measured daily during and after administration of the drugs. The survival time was observed and the haematocrite values were also determined. The activity of the antioxidant enzymes was also measured during the course of the infection. (I) The MAP-1987 alone was not toxic even in a high concentration (2500 µg/kg) and did not change the survival time (7 days); (II) the chloroquine administered alone stabilized the parasitaemia at a low level (16%) and increased the survival time (14 days); (III) the combined administration of the MAP-1987 and chloroquine cured the mice suppressing totally the parasites; (IV) the combined treatment increased the activity of the antioxidant enzymes in the blood cells of the infected animals.

G. DREYFUSS and PH. LOISEAU

Experimental African trypanosomiasis of the sheep: properties of the model for plasma kinetics of some trypanocidal compounds*Laboratory of Parasitology, Faculty of Pharmacy, Limoges, and Laboratory of Biology and Control of Parasitic Organisms, Faculty of Pharmacy, Chatenay Malabry, France*

1. The research for new trypanocidal compounds is carried out on two chemical series: metallic complexes derived from Iridium and Rhodium, and organoarsenical compounds. Concerning metallic complexes, the recent discovery of the high trypanocidal properties of Ir-COD-pentamidine has led to the synthesis of analogues whose the structural modifications are the following: (M-X-pentamidine) Y; where M = Ir, Rh, X = Cyclooctadiene (COD), cyclooctatetraene (COT), Y = NO₃, ethylfumarate, alizarine red, B(C₆H₅)₄.

2. The primary screening is in development in vitro and in vivo on *Trypanosoma brucei brucei* IPP, fast strain. The preliminary results show an influence of the salt on the biological activity. This aspect will be studied more precisely by

kinetics studies. Concerning the arsenicals, about 120 spiroarsoranes have been synthesized and evaluated for their trypanocidal properties in vitro and in mice. The most promising compound of this series was active at $150 \mu\text{mol/kg}^{-1}$ subcutaneously administered in one single dose. The "spiranization" of arsonic acids led to the spiroarsorane structure which can be seen as a drug carrier since the hydrolysis of spiroarsoranes release arsonic acids.

3. The use of the *T. brucei brucei* sheep model, perfected by our colleagues in Limoges (France), allows us to evaluate some interesting trypanocidal compounds on the lymphatic-plasma phase of the disease. In the same experience, we have studied the plasma pharmacokinetics of platinum of two cis-Pt pentamidine salts (chloride and iodide), and that of a spiroarsorane derivative. Both chloride and iodide salts of cis-Pt pentamidine complexes have shown a trypanocidal activity in sheep, after respectively a single subcutaneous injection of 12.5 and 5 mg/kg^{-1} . Spiroarsorane derivative was active after a 30 mg/kg^{-1} injection. The three compounds were distributed quickly and largely within deep compartments according to a monocompartmental model.

J. O. OLOBO and J. I. GITHURE

A monkey model for anti-leishmania vaccine and drug development

*Institute of Primate Research, National Museum of Kenya, and Kenya Medical Research Institute,
Biomedical Research Centre, Nairobi, Kenya*

Leishmaniasis is a disease that results from interaction by several species of digenetic protozoan parasites *Leishmania*. Promastigote forms of this parasite are found in the vector sandflies and in vitro. Following the bite of the infected sandfly the promastigotes are released into the host tissues where they invade the macrophages before transforming into amastigotes. Since non-human primates are phylogenetically closer to man, the of a species in which the disease spectrum resembled the human situation would be an ideal model for both human vaccine and anti *Leishmania* drug development.

The vervet monkey (*Cercopithecus aetiops*) has been shown to be naturally infected with *Leishmania major* (cutaneous leishmaniasis) in our laboratory. Subsequent studies to establish the validity of the disease model demonstrated that both infected phlebotomes sandfly bite on needle inoculation of culture-derived promastigotes resulted in infection and eventual self-cure. Infection with *Leishmania donovani* (visceral leishmaniasis) via the needle resulted in either death or self-cure, cryptic or subclinical infections. There was no major alteration in the haemogram values following *L. major* infection. However, infection with *L. donovani* resulted in leucopaenia and anaemia. Animals that died following infection had hepatosplenomegaly and histopathology demonstrated mononuclear cell infiltration. In addition, *L. donovani* infected animals had increased levels of alkaline phosphatase, hypoalbuminaemia and hyperglobulinaemia of varying severity. As further

pathological and clinical events are studied during experimental infection with the two species of *Leishmania* it becomes clearer that the vervet monkey resembles the human situation during infection with either of the parasites. We conclude that this animal species would be a valuable model to study the different aspects of both visceral and cutaneous leishmaniasis and would be useful to screen potential anti-leishmanial drugs for clinical application.

J. EUZEBY

**Therapy of the generalized leishmaniasis of dogs;
actual situation, and perspectives**

School of Veterinary Medicine of Lyon, Lyon, France

Following the brief summary of the opportunities of anti-leishmania therapy, the author defined the basic features of specific treatment, and reviewed the different possibilities of the application of therapeutics. Stibium derivatives, and aromatic diamidines are the favourite drugs applied at present, nevertheless, numerous other chemicals and also antibiotics possess anti-leishmania activities. These combinations, and also the classical drugs should be tested under experimental therapeutic conditions in the form of liposome preparations, since these may provide more precise access of the drugs to the target parasites, applying lower doses, and better tolerance of the side effects. The indications of palliative therapy, including the possibilities of the control and monitoring of the specific treatment, are summarized.

J. MOLNÁR, Y. MÁNDI, J. BARBE and I. OCSOVSZKY

Effects of acridines on bacteria

Institute of Microbiology, and Institute of Biochemistry, Albert Szent-Györgyi University Medical School, Szeged, Hungary, and GERCTOP, Faculty of Pharmacy, University of Marseille, Marseille, France

Different amino- and imino-acridines were systematically synthesized. The antibacterial, antiplasmodial, antimitotic and endotoxin complexing effect of BG204-diethylamino-ethylthio-9-acridine, BG443-aminobenzyl-dibenzyl-9-amino-acridine, BG445-2,2'-(Bis-N,N-)-3-aminoethylamino-9-dimethylamino-propyl-thio-acridinone-biphenyl-dibromhydrate, BG31A-N-buthylamino-A, BG31i-N-buthylamino-9-imino-A, BG35A-N-heptyl-amino-A, BG35i-N-heptyl-9-imino-A, BG37A-N-propylamino-A, BG37i-N-propyl-9-imino-A, BG39A-N-ethylamino-A, BG39i-N-ethyl-9-imino-A, BG41A-N-diethylamino-ethyl-A, BG41i-N-dimethylamino-ethyl-9-imino-A, BG42A-

N-diethylamino-propyl-A, BG42i-N-diethylamino-propyl-9-imino-A, BG43A-N-isobuthylamino-A, BG43i-N-isobuthyl-9-imino-A were studied.

The antibacterial effect of the compounds was compared on *Escherichia coli*. Amino-derivatives were more active than imino-acridines. The N-heptyl-9-imino-acridine was able to select clon minus mutants in the *E. coli* culture, however, the others were ineffective in this respect. The imino-acridines inhibited the motility of *Proteus vulgaris* more effectively than amino-derivatives. This antimotility action of the acridines was dependent on the ionic content of the media.

The antiplasmid effect was measured on F-prime plasmid of *E. coli* LE140 strain. Imino-acridines had more powerful antiplasmid effect than the amino-substituted derivatives. The majority of the compounds inhibited the intracellular plasmid transfer from *E. coli* Km^r donor to Na-azid resistant recipient. In this test the amino-substituted derivatives were shown to be more effective than the imino-substituted compounds.

Endotoxin formed complexes with the N-buthylamino- and N-propylamino- and imido-derivatives. However, complex formation of N-ethyl-, N-heptyl-, N-diethylamino-ethyl- and N-diethylamino-propyl-acridines were different.

Y. MÁNDI, K. RÉGELY, I. OCSOVSKY, J. BARBE and J. MOLNÁR

Effect of acridines on the tumour necrosis factor production by human leukocytes

Institute of Microbiology, and Institute of Biochemistry, Albert Szent-Györgyi University Medical School, Szeged, Hungary, and Faculty of Pharmacy, GERCTOP, University of Marseille, Marseille, France

Serum Tumour Necrosis Factor (TNF) level has been found to be elevated in patients suffering malaria. The investigation of the effect of some acridine derivatives on the production of TNF might be important. Different acridines were systematically synthesized and the effect of these compounds was tested on endotoxin and *Staphylococcus aureus* induced TNF production by human leukocytes. Iminoacridines as 31 A (N-butyl-amino-acridine) and 39 I (N-ethyl-9-imino-acridine) totally abrogated TNF production of leukocytes at a concentration of 1.25 µg/ml, while their variants 31 I (N-butyl-9-imino-acridine) and 39 A (N-ethyl-amino-acridine) exerted only a 50% inhibition at the same concentration. Derivatives signed as 41 A (N-dimethylamino-ethyl-acridine) and 41 I (N-dimethylamino-ethyl-9-imino-acridine) exerted only a moderate 30 and 10% inhibition at a concentration of 2.5 µg/ml, respectively. No significant modulation of TNF production was observed applying acridines of the series signed BG (heptylamino-, propylamino-, isobuthylamino-, and diethylamino-propyl derivatives of acridines). The TNF mediated cytotoxic effect of monocytes against WEHI cells was reduced as well by the most effective compounds. The acridines did not interfere with the expression of CD 14 molecules on monocytes. The

exact mechanism of the suppression of TNF synthesis by acridines has to be elucidated but might be useful in the evaluation of their antimalariatic properties.

I. B. PETRI, I. BEREK, J. BARBE and J. MOLNÁR

**Immunomodulating effect of acridines on prokaryotic
and eukaryotic cells**

Blood Transfusion Centre, and Institute of Microbiology, Albert Szent-Györgyi University Medical School, and University of Food Industry Horticulture Institute, Szeged, Hungary, and Faculty of Pharmacy, GERCTOP, University of Marseille, Marseille, France

It is known that the most specific agents against trypanosomes and malaria parasites are antibodies produced by the immune system, however, this is the less effective mechanism *in vivo*, because immunosuppression occurs in both types of severe disease. In addition, plasmodia are not particularly antigenic and like the trypanosomes, exhibit the disturbing phenomenon of antigenic variations, leading further difficulties in the host-immune response.

Considering these immunosuppressive effects of the parasitic infections and the present therapeutic possibilities, the newly synthesized substituted acridine derivatives were screened for immunomodulating effects. Different acridines were systematically synthesized as follows: B204 diethylamino-ethylthio-9-acridine; BG443-aminobenzyl dibenzyl-9-aminoacridine; BG445 2,2-(Bis-N,N-)-3-aminoethylamino-9-dimethylamino-propyl-thio acridine(-)-biphenyl dibromhydrate; BG31A N-buthylamino-A; BG31i N-buthyl-9-imino-A; BG35A N-heptylamino-A; BG35i N-heptyl-9-imino-A; BG37A N-propylamino-A; BG37i N-propyl-9-imino-A; BG39A N-ethylamino-A; BG39i N-ethyl-9-imino-A; BG41A N-dimethylamino-ethyl-A; BG41i N-dimethylamino-ethyl-9-imino-A; BG42A N-diethylamino-propyl-A; BG42i N-diethylaminopropyl-9-imino-A; BG43A N-isobuthylamino-A; BG43i N-isobuthyl-9-imino-A.

Several immunological effects of the compounds were tested by examining ADCC (antibody dependent cellular cytotoxicity) and blast transformation of lymphocytes. The drugs had a suppressive effect at 5 µg/ml concentration and showed a higher level in the case of imino-acridines. After the *in vitro* screening the acridines with immunosuppressive effects can be excluded from further *in vivo* studies, however, as acridine derivatives simultaneously exert antimalarial and immunostimulating effect may be recommended as the drugs of the future for treatment of malaria.

R. PUSZTAI, J. MOLNÁR and N. MOTOHASHI

Effect of benz(c)acridine on frameshift mutation and adenovirus infection

*Institute of Microbiology, Albert Szent-Györgyi University Medical School, Szeged, Hungary, and
Department of Medicinal Chemistry, Meiji College of Pharmacy, Tokyo, Japan*

The mutagenicity of various benz(c)acridine derivatives was tested on *Salmonella typhi-murium* TA-98 His⁻ strain in Ames test. The mutagenicity index (m.i.) of 5,7-dimethyl-benz(c)acridine was the lowest, 32. The other compounds: 7-methyl-, 7,9-dimethyl-, 7,11-dimethyl- and 7,9,11-trimethyl-benz(c)acridine showed higher m.i., 424, 237, 59 and 204, respectively. The effect of the same compounds was tested on oncogene (E1) expression of human adenovirus in HEP-2 cells by means of immunofluorescence. The highly mutagenic compounds had little or no effect on oncogene expression, however, 5,7-dimethyl-benz(c)acridine increased the number of cells containing oncogene product. The activity of 5,7-dimethyl-benz(c)acridine on adenovirus infection seems to depend on the simultaneous charge distribution in the K- and L-regions of the molecule as a consequence of hyperconjugation on the 5th and 7th C atoms. The reduced mutagenic activity of this compound can be explained by the high charge density at the L-region of the molecule.

L. MAES, E. B. SONGA and R. HAMERS

Dithiarsepines, a new series of trypanocides

Institute for Molecular Biology, Vrije University of Brussel, St. Genesius Rode, Belgium

The new trypanocidal molecule IMOL 881, synthesized at our laboratory, has been evaluated in more detail using several animal models. Mice and rabbits, infected with the strain ROTAR 1 of *Trypanosoma evansi* were fully cured, even at a high parasitaemia, by a single i.m. drug injection. High parasitaemias induced in mice by virulent stocks of *Trypanosoma gambiense* and *Trypanosoma rhodesiense* were also eliminated by a single treatment. Goats submitted to a long-term *T. gambiense* infection, provoking a late stage trypanosomiasis, were cured, too. The drug's potential for an efficient treatment of CNS stage trypanosomiasis was confirmed by the experiments with Dr. Jennings' mouse model specifically designed for this purpose. Test results on mice which were pretreated with the drug and infected subsequently with *T. evansi*, show that the product also exhibits prophylactic properties. Preliminary acute toxicity tests have indicated that the drug is well tolerated, even at the highest level tested (500 mg/kg).

Overall, the new trypanocide seems to offer the combination of a good and polyvalent curative activity with a low toxicity, resulting in a high therapeutic index.

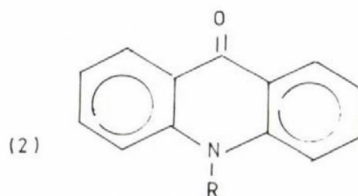
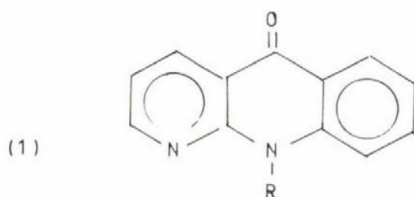
Chemically, the compound belongs to a new series of dithiarsepanes. Its mode of action probably involves a multiple interference, e.g. with the lipoic acid and the trypanothione metabolism of the trypanosome which was found to exist for related organoarsenicals (A.H. Fairlamb). Some of the new derivatives being synthesized will probably offer the additional benefit of an interesting economical potential, also enabling their use in animal trypanosomiasis.

J. J. CHARBIT, H. MEFETAH, A. M. GALY, J. P. GALY, J. BARBE, M. PLACIDI,
J. J. CASTILLA-CALVENTE, C. MESA-VALLE and A. OSUNA

Naphthyridones and acridinones as trypanocidal agents

GERCTOP - URA CNSR 1411, Laboratory of Chemotherapy, Faculty of Pharmacy, Marseille, France,
and Department of Parasitology, University of Granada, Granada, Spain

Synthesis of some new derivatives belonging to the title-series have been achieved. Synthetic pathways are portrayed. Naphthyridones (1) and acridinones (2) are quite similar from a structural point of view. This has been demonstrated by conformational studies using Molecular Mechanics (Maximim II - Sybyl software).



However, naphthyridones are more effective than homologous acridinones against *Trypanosoma cruzi* strain "in vitro", as shown in the Table.

Compounds	Inhibitory effects on parasite growth Doses ($\mu\text{g/ml}$)			LD ₅₀ i.p. (mg/kg)
	100	10	1	
1a	100	100	85	120
2a	60	0	0	250
1b	100	95	90	50
2b	100	0	0	420

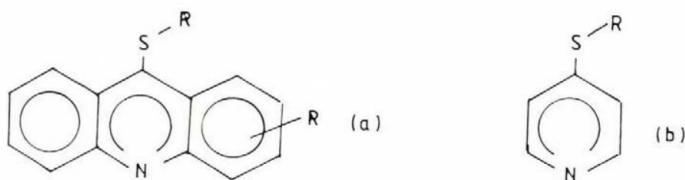
Finally, acute toxicity has been estimated in IOPS mice with a view to select test-sample compounds for further investigations.

A. M. GALY, C. JOHNSON, A. MAHAMOUD, J. P. GALY, J. BARBE,
J. J. CASTILLA-CALVENTE, C. MESA-VALLE, A. OSUNA, S. DRINNAN
and D. SHARPLES

SAR in the field of trypanocidal acridines

GERCTOP - URA CNRS 1411, Marseille, France, Department of Parasitology, University of Granada,
Granada, Spain, Department of Pharmacy, Manchester, UK

Thioacridine compounds (a) have demonstrated a good trypanocidal activity against *Trypanosoma cruzi* strains "in vitro".



With respect to this, the most convenient side chain (R') to be branched is the dialkylaminoalkyl one. However, nothing was certain about the role of the heterocyclic moiety. Hence, with a view to clear this role, some 4-(thio-substituted)-pyridine compounds (b) were prepared and tested. Syntheses were achieved alkylating 4-mercapto pyridine under reflux in ethanol/soda. Compounds isolated, were characterized by NMR spectroscopy.

Drugs were tested during exponential growth phase of parasites. The *T. cruzi* strain was isolated from a clinical case (Institute of Malariology in Macaray, Venezuela). Epimastigote forms of the parasite were cultured in liquid *Trypanosoma* medium at 28 °C. Assays were performed in multiwell plates. The binding of drugs to DNA was spectrophotometrically measured by the effect of DNA on UV spectra of derivatives. All measurements were carried out in 0.018 mol/l Tris Cl buffer (pH=7.0). Binding constants were calculated using "Enzfitter", a non linear regression data analysis Programme from Elsevier Biosoft. On the one hand, there are no correlations between intercalation into DNA and activity under examination. On the other hand, pyridyl moiety does not carry any activity, so that one must admit that the central ring of thioacridines is not capable by its own to induce trypanocidal activity.

SZ. NAGY, J. MOLNÁR and J. BARBE

Antiproliferative effect of amino- and imino-acridines

*Institute of Microbiology, Albert Szent-Györgyi University Medical School, Szeged, Hungary, and
GERCTOP, Faculty of Pharmacy, University of Marseille, Marseille, France*

The systematically synthesized amino- and imino-acridines: BG204-diethylamino-ethylthio-9-acridine, BG443-aminobenzyl-dibenzyl-9-amino-acridine, BG445-2,2'-(Bis-N,N-)-3-aminoethylamino-9-dimethylamino-propyl-thio-acridinone-biphenyl-dibromhydrate, BG31A-N-buthylamino-A, BG31i-N-buthylamino-9-imino-A, BG35A-N-heptylamino-A, BG35i-N-heptyl-9-imino-A, BG37A-N-propylamino-A, BG37i-N-propyl-9-imino-A, BG39A-N-ethylamino-A, BG39i-N-ethyl-9-imino-A, BG41A-N-dimethylamino-ethyl-A, BG41i-N-dimethylamino-ethyl-9-imino-A, BG42A-N-diethylamino-propyl-A, BG42i-N-diethylamino-propyl-9-imino-A, BG43A-N-isobuthylamino-A, BG43i-N-isobuthyl-9-imino-A were tested on proliferation of HEp-2 cells. The compounds BG31A, 31i and 42A inhibit the multiplication of the cells at 20 µg/ml, however, other compounds did at 50-120 µg/ml. We suppose that the hydrophobicity or the charge of the side chain plays an important role in the antiproliferative effect of substituted acridines.

A. CREMIEUX, J. CHEVALIER, H. BERNY, A. M. GALY, P. BROUANT,
J. P. GALY and J. BARBE

**Evidence that acridine derivatives cause lysis
of *Escherichia coli***

*Laboratory of Microbiology, Faculty of Pharmacy, Marseille, France, Department of Biology, University
Ibn Tofail, Kenitra, Morocco, and GERCTOP - URA CNRS 1411, Faculty of Pharmacy, Marseille, France*

The study of macromolecule biosynthesis inhibition by some new 9-acridinone and 9-thioalkoxy-acridine derivatives showed a similar and very low rate of all precursors incorporation at the highest concentrations studied. Cell lysis was suspected and demonstrated by the decrease in counts of *Escherichia coli* cells. Evidence of lysis was confirmed by electron microscopy studies and by tests with sheep erythrocytes. This phenomenon is still under investigation.

J. CHEVALIER, A. CREMIEUX, H. BERNY, A. M. GALY, P. BROUANT,
J. P. GALY and J. BARBE

**Investigation on the antibacterial mechanism of action of
new acridine derivatives**

*Laboratory of Microbiology, Faculty of Pharmacy, Marseille, France, Department of Biology, University
Ibn Tofail, Kenitra, Morocco, and GERCTOP - URA CNRS 1411, Faculty of Pharmacy, Marseille, France*

New 9-acridinones and 9-thioalkoxyacridines with antimicrobial activities have been studied. Inhibitory effects on DNA, RNA and protein synthesis in *Escherichia coli* cells have been examined. Results showed that all drugs inhibit the biosynthesis of macromolecules, but in different ways. RNA synthesis which is clearly decreased 5 min after exposure of cells to drugs was equally lowered during the 60 min experiment. In contrast, inhibition of DNA synthesis, generally detected at 5 min time but at a lower extent than inhibition of RNA synthesis, decreased after 15 or 30 min. In most cases, protein synthesis was affected later than RNA and DNA synthesis and the maximum rate of inhibition was noted at time 60 min. In accordance with these results, inhibition of RNA synthesis may be considered as the main step involved in the antimicrobial activity of these drugs.

C. MESA-VALLE, J. J. CASTILLA-CALVENTE, N. RODRIGUEZ, T. ARNEDO,
V. MORALEDA, J. BARBE and A. OSUNA

**Inhibitory effects of acridine derivatives BG-203, BG-237
and BG-289 on the growth of amastigote forms of
Trypanosoma cruzi obtained in vitro**

*Investigation Group of Molecular Biochemistry and Parasitology, University of Granada, Granada, Spain,
and Laboratory of Inorganic Chemistry, Faculty of Pharmacy, University of Marseille, Marseille, France*

Trypanosoma cruzi, a parasite protozoon belonging to the order *Kinetoplastida* and the family *Trypanosomatidae*, is the pathogen responsible for American trypanosomiasis, a disease for which there is currently no effective chemotherapy available. In the present work, the activity of the acridine derivatives BG-203, BG-237 and BG-289 have been evaluated in treatment against amastigote forms of *T. cruzi* obtained in vitro. The amastigote forms were obtained from metacyclic forms of the parasite by heat-shock, and cultured on TC-199 medium at 37 °C. These forms obtained in vitro are morphologically, biochemically and antigenically similar in the aflagellate intracellular forms of *T. cruzi*. When the growth rate was 1×10^6 parasites/ml, the effects of the three acridine derivatives were assayed at concentrations of 100, 10 and 1 µg/ml, at 24, 49 and 72 h from the initial treatment. The activity of the all three derivatives was found to be high, exceeding 70% inhibition at the

concentration of 100 µg/ml at 24 h; 100% growth inhibition was reached at a concentration of 10 µg/ml at 72 h after treatment.

J. J. CASTILLA-CALVENTE, C. MESA-VALLE, N. RODRIGUEZ, J. LAZUEN,
V. MORALEDA, J. BARBE and A. OSUNA

**Analysis of the preventive effect of the newly synthesized
acridine derivatives BG-203, BG-237 and BG-289
on blood banks infected with *Trypanosoma cruzi***

*Investigation Group of Molecular Biochemistry and Parasitology, University of Granada, Granada, Spain,
and Laboratory of Inorganic Chemistry, Faculty of Pharmacy, University of Marseille, Marseille, France*

American trypanosomiasis, or Chagas disease, is endemic in Central and South America, while in North America only isolated cases have been detected.

The pathogen, *Trypanosoma cruzi*, is a haematic flagellate protozoa (family *Trypanosomatidae*, order *Kinetoplastida*) against which there is currently no effective chemotherapy available. For this reason, a long list was compiled and the compounds tested in vitro/in vivo for therapeutic effectiveness against American trypanosomiasis, although, in general the effectiveness of the treatments has been limited, the toxicity excessive, or the administration requirements troublesome. In the present work, blood was extracted from BALB/c mice infected with *T. cruzi*, and was kept refrigerated before being treated with the compounds BG-203, BG-237 and BG-289, products chosen after broad screening for their low toxicity and effectiveness against in vitro epimastigote and metacyclic forms of the parasite. After treating the blood with three different doses of the compounds for 4, 7 and 21 days, respectively, the mice were re-infected and analysed for the effects on the parasitism index. These parasitism indices showed clear decreases after treatment with the different compounds.

A. MONESTIER MORALES

**Activity of five acridine derivatives against *Phytomonas*
isolated from the coconut**

Investigation Group of Molecular Biochemistry and Parasitology, University of Granada, Granada, Spain

The genus *Phytomonas* (family *Trypanosomatidae*) include plant parasites which cause serious damage to crops – principally tropical crops such as the coconut – by producing a lethal and up to now irreversible disease called “Hartrot”. In the present study, five acridine derivatives have been evaluated as treatments against Hartrot. These products were chosen after abroad screening of acridine derivatives used to treat other *Phytomonas* isolated from *Euphorbia characias*; in this plant these five

compounds produced significant inhibition levels. We have quantified, for different dosages and incubation times, the percentage of inhibition that these five acridine derivatives produced against in vitro cultures of *Phytomonas* Hartrot. In addition, we have attempted to determine the alterations caused by these compounds at the cellular level, as well as the possible impact of the treatments of the energy metabolism of the parasite.

C. MESA-VALLE, J. J. CASTILLA-CALVENTE, M. SANCHEZ-MORENO,
J. LAZUEN, N. RODRIGUEZ, V. MORALEDA, J. BARBE and A. OSUNA

**Activity of the acridines BG-203, BG-237, BG-289,
BG-325 and BG-374 in the interiorization in HeLa cells
of pretreated metacyclic forms of *Trypanosoma cruzi***

*Investigation Group of Molecular Biochemistry and Parasitology, University of Granada, Granada, Spain,
and Laboratory of Inorganic Chemistry, Faculty of Pharmacy, University of Marseille, Marseille, France*

The effects of five newly synthesized acridine derivatives have been studied in the interiorization process of the infective metacyclic forms of *Trypanosoma cruzi* on HeLa cells pretreated with these compounds. The metacyclic forms of the parasite were obtained in vitro from the epimastigote forms cultivated in GRACE medium for 8 days at 28 °C. The HeLa cell line used was isolated by Gey et al. from a human cervix tumour in 1950; the cells used in our experiments came from a strain maintained in the Cytological Research Institute of Valencia, Spain, in 1975. The HeLa cells were pretreated with the acridine derivatives BG-203, B-237, BG-289, BG-325 and BG-374 for 6 h at a concentration of 1 µg/ml. Afterwards the cells were infected with the metacyclic forms of the parasite, and at 24, 48 and 72 h after infection the parasitism rates were determined. All the acridine derivatives reduced the percentage of parasitism and the infection index, beginning 24 h after treatment.

J. J. CASTILLA-CALVENTE, C. MESA-VALLE, M. SANCHEZ-MORENO,
T. ARNEDO, N. RODRIGUEZ, V. MORALEDA, J. BARBE and A. OSUNA

**Ultrastructural study of the action of the acridine
derivatives BG-203, BG-237 and BG-289 on HeLa cells**

*Investigation Group of Molecular Biochemistry and Parasitology, University of Granada, Granada, Spain,
and Laboratory of Inorganic Chemistry, Faculty of Pharmacy, University of Marseille, Marseille, France*

We have evaluated the in vitro effect of three newly synthesized acridine derivatives on HeLa cells. These studies are part of an effort by our laboratory to find an effective chemotherapy, unknown at the moment, against Chagas disease and Kala-

azar, the infectious agents of which are *Trypanosoma cruzi* and *Leishmania donovani*, respectively. After a prior in vitro screening, the acridines BG-203, BG-237 and BG-289 showed the greatest anti-parasite activity, and the least cytotoxicity. With these data, we began to determine the ultrastructural effects these derivatives might have on HeLa cells. The cell line was isolated in 190 from a human cervix and maintained in our laboratory, cultured on MEM medium supplemented with 20% IFCS. The HeLa cells, cultivated at 37 °C, were treated for 8 h with a concentration of compound at 10 µg/ml. After this time the supernatant was removed, the cells separated from the culture flask and submitted to the normal process for examination under the transmission electron microscope. A study of the sections revealed the effects of the derivatives at the nuclear and especially the mitochondrial level, showing characteristic lipid vacuoles and membranous circumvolutions.

P. KAGERUKA and S. GEERTS

Research on chemotherapy for trypanosomiasis

Institute of Tropical Medicine, Veterinary Department, Antwerpen, Belgium

Animal trypanosomiasis remains one of the most important diseases as a major impediment to livestock production in sub-Saharan Africa (Tsetse transmitted trypanosomes), North-Africa, South-America (no tsetse transmitted trypanosomes). Whereas, human trypanosomiasis, either African sleeping sickness or Chagas' disease in South America is still a significant public health problem. Until now chemotherapy played a vital role in maintaining and improving the production of domestic livestock and controlling human trypanosomiasis. However, at present there are only a few drugs available for therapeutic and prophylactic use against the disease and for some of them significant problems with drug resistance are being reported from many parts of the World. In light of this situation there is an urgent need (I) for the development of new trypanocides, especially drugs that are chemically unrelated to those currently on market; (II) to ascertain the factors affecting the use of currently available trypanocides; (III) to reduce systemic and local toxicity and to obtain a longer and more reliable prophylactic period of drugs.

Research carried out in our laboratory concern:

(I) The standardization of posology of currently available trypanocides on laboratory animals. The on going research concerns homidium bromide (Ethidium) and isometamidium chloride (Samorin). The objective of this work is to develop a tool for the evaluation of trypanocidal activity and to demonstrate the chemo-resistance. The tests were carried out in vivo on mice model and in vitro/in vivo by incubation technique. The results obtained show the difference of sensitivity between the stocks of *Trypanosoma congolense* at the recommended therapeutic and prophylactic doses (Kageruka et al., in preparation). The in vitro cultures of procyclic and bloodstream forms is possible and is envisaged in near future. To undertake this work we have

cryopreserved well documented stocks of trypanosomes from different geographical areas.

(II) The reinforcement of prophylactic effect of homidium bromide (Ethidium) and isometamidium chloride (Samorin) is studied by use of polymeric slow release device. The results obtained in *T. congolense* infection following the subcutaneous implantation of coated copolymer containing 1 mg/kg homidium bromide or isometamidium chloride have better and improved prophylactic activity than intramuscular injection.

(III) The therapeutic and prophylactic trials on the currently available molecules on the market, whose known trypanocidal activity has not been studied thoroughly. For this purpose we have used ronidazole (5-nitroimidazole) administered parenterally and orally (in water or feed pellets) to rats and rabbits infected experimentally. The results obtained as therapeutic and prophylactic treatment are promising against infections with *T. congolense*, *T. brucei* and *T. evansi*. In future experiments, association of ronidazole with other trypanocides with potentiation or/and synergistic effects could provide more interesting results in the treatment of chronic form of *T. brucei* sp.

(IV) In the framework of "COST ACRIVAL" our contribution could be in the chapter "Parasitology" – in vivo and in vitro trials and in the chapter "Toxicology", in collaboration with an ad hoc laboratory.

LETTER TO THE EDITOR

HLA-DR β , -DQ β DNA POLYMORPHISMS IN 9 JUVENILE RHEUMATOID ARTHRITIS AFFECTED HUNGARIAN CHILDREN

(Received June 7, 1993)

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To the Editor:

Earlier population and family studies have shown that genetical and environmental factors are equally important in the development of the multifactorial disease: juvenile rheumatoid arthritis (JRA). The genetical component of the disease might be also complex and probably several genes are involved in the ethiology. In our days we know more about genetical sensitivity than of the causative agent. Chatila et al. [1], Merling et al. [2], Maksymowych et al. [3], Fernandez-Viña et al. [4] have shown the frequent presence of DR5, w8, DRw6 genes in the pauciarticular form of JRA but the "sensitivity" gene and the "epitop's" exact structure has not yet been determined. We would provide additional data in examining 9 JRA patients' DR β , DQ β genes with several RFLP studies using the Taq I, Bam H I, Bgl II, Eco RI, Hind III, Pst I, Pvu II restriction endonucleases in standard Southern-blot hybridization methods. We applied the following cDNS probes: p-II-beta-1 (DQ β) and p-II-beta-3 (DR β) (gifts from Ann-Kristin Jonsson, Uppsala). The DR β , DQ β genotyping was done according to the data of Bidwell et al. [5, 6] and Carlsson et al. [7]. Using the Taq I DR β RFLP the identification of DRw 53, 52 a, b supertypes is also possible [8]. The use of more restriction enzymes and hybridization with the DR β , DQ β probes was done to generate data for the separation of the 11¹, 11² and 13a⁴, 14a (DR5 and DRw6 subtypes) in the DQ β region (linkage disequilibrium). Our results are summarized in Table I.

There was no difference among our patients according to sex ratio. We found DRw53 supertype in 2 of our patients, one was DRw52a positive and 6 (67%) were DRw52b positive. The occurrence of the DRw52b positivity is three times higher compared to the normal population. Two patients were found DRw52b homozygotes (No 8, 9) and only these two patients were serum antinuclear factor seropositives. We found that out of our 1 systemic, 1 pauciarticular and 7 polyarticular JRA patients, these, who were DRw52b positive were clinically more serious cases. Only one of our patients had irido-cyclitis. In contrast to the results of Vicario et al. [9] we found in 8 out of our 9 patients the 2.9 kb length fragment positivity with Eco RI/DR β RFLP and also at their family members' analysis, so we do not consider the presence of this fragment being a disease marker. In accordance to Fernandez-Viña et al. [4] we could not detect the DR 4 (Dw4, 14) sensitivity antigens seen frequently in adults'

rheumatoid arthritis. Our results point to the possibility of a common epitop in JRA and underline the importance of the study of the HLA-class II gene polymorphism in the understanding of the genetical background of the sensitivity for the disease.

Table I

HLA-DR_B -DQ_B genotyping of our juvenile rheumatoid arthritic patients

N	Patients, (age), sex**	HLA alleles			ANF*	Clinical course		
		DRB1	DRB3/DRB4	DQB1		form	severity scores***	persist- time
1	V. Cs. (13 yr) m	1,	-	1a,	-	systemic	I	4 yr
2	Á. I. (17 yr) m	7 ¹ , 11 ²	52a, 53	1b, 2b	-	polyarticular	I	6 yr
3	H. B. (16 yr) m	7 ¹ , 8	-	53	-	- " -	II	12 yr
4	B. Á. (8 yr) f	8, 11 ¹	-	52b	-	- " -	II	5 yr
5	N. Sz. (20 yr) f	1, 11 ¹	-	52b	-	- " -	III	9 yr
6	K. A. (15 yr) f	1, 11 ¹	-	52b	-	- " -	IV	8 yr
7	B. A. (6 yr) f	10, 11 ¹	-	52b	-	- " -	IV	3 yr
8	R. K. (17 yr) m	14a, 17 ²	52b, 52b	1a, 2a	+	- " -	IV	8 yr
9	G. A. (5 yr) f	11 ¹ , 17 ²	52b, 52b	2a, 3b	+	pauciarticular	III	3.5 yr

*ANF = antinuclear factor

**sex:

m = male

f = female

***Severity of the disease:

I, no signs more than 6 mo/yr;

II, no signs less than 6 mo/yr;

III, symptoms in spite of therapy;

IV, articular deformities

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BOOK RECEIVED

G. I. Barrow and R.K. A. Feltham (eds): *Cowan and Steel's Manual for the Identification of Medical Bacteria*. Third Edition. Cambridge University Press 1993. pp. 331. Price £ 40.00

In the recent 25 years the Cowan and Steel manuals have given a helping hand for the clinical bacteriologists in the identification of bacteria. The new edition has been edited also with thoroughness and demanding care as we are accustomed to it. This book is made not only in the light of the new taxonomical data, but also contains the traditional and modern diagnostic methods which are essential in the identification of medically important bacteria.

In the different manuals rarely retraceable "Minidefinition" part of the book is very useful especially in the everyday practice. The identification tables comprise another important part, these were characteristic features of the former editions, too. The only question is – in connection with the tables – would not have been better to use the percentage of positive results instead of "+" "-" and "d" in the case of taxons represented by a sufficient number of isolates. The readers of the manual will probably be mostly specialists and they could use those data better without any confusion. Regarding the spelling of authors' name, in this edition some errors may be noted: Voges-Proskauer (sic) test, Lowenstein-Jensen (sic) medium; the correct names being Proskauer and Löwenstein (or Loewenstein).

To sum it up the third edition of Cowan and Steel's is a very useful reference book and it is essential for all the clinical bacteriologists all over the world.

A. Lipcsey

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IMMUNOLOGICAL ASPECTS OF THE EFFECT OF PENTOXIFYLLINE (TRENTAL)

(A BRIEF REVIEW)

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(Received July 29, 1993)

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Pentoxifylline (methixanthine derivate) has haemorheological activity, thought to be based on its ability to reduce blood viscosity and increase the filterability of blood cells and widely used for the treatment of various types of vascular insufficiency. Recent studies provide evidence for the effect of this drug on immune functions. Pentoxifylline is able to influence *in vitro* as well as *in vivo* production of cytokine including tumour necrosis factor α . This drug has been shown to attenuate the symptoms of endotoxin shock and adult respiratory distress syndrome. Its beneficial effect on tumour-induced cachexia, organ transplantation and AIDS can widen the indications of clinical administration. Nevertheless, the chronic continuous administration of Pentoxifylline in arterial insufficiency is to be considered due to its potentially enhanced susceptibility to malignant disorders.

Introduction

Pentoxifylline (Ptx) has been widely used for the treatment of peripheral arterial insufficiency and its clinical efficacy has been proved, however, the proposed mechanism of this drug was questioned by some authors for the following reasons. First, an impaired erythrocyte deformability has not consistently been observed in claudicants. Second, the incubation times and drug concentrations required to change red cell filterability were doubtful in relevance to the clinical use of this drug. Third, one of the known biochemical effects of the drug, phosphodiesterase inhibition, suggests to us more likely that Ptx might work its benefit through effects on polymorphonuclear granulocytes (PMNs), mononuclear cells as well as platelet functions or both, rather than an effect on a mild and inconsistently observed abnormality in red blood cell filterability [1–3]. Moreover, the inhibition of phosphodiesterase is unlikely to explain the complex effect of Ptx on PMNs and mononuclear cells for at least two reasons. First, theophylline has been proved to be a

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less potent inhibitor of metabolic activity of PMNs, although both Ptx and theophylline are very close to equipotent in elevating cAMP levels in cells [4]. Second, the levels of cAMP achieved with even 500–1000 µg/ml of Ptx are far below those reported by Confer and Eaton to be required for inhibition of chemotaxis and superoxide production of PMNs [5].

Recently, there is an accumulating evidence of Ptx for its immunomodulatory, anti-inflammatory effects that might provide new indications of this drug in clinical use. The mechanism by which Ptx can exert its effect on immune processes is multifaceted, involving regulation of the cytokine network [6–8].

Effect of Ptx on the cytokine network

The growing knowledge of the physiological and pathophysiological role of cytokines in acute and chronic inflammatory processes as well as neoplastic disorders stimulated efforts to control their synthesis and actions pharmacologically in clinical situations. Tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1) have been shown to be important mediators of fever. Ptx has been shown to suppress the synthesis of TNF- α induced by endotoxin in cell cultures as well as experimental animals [9]. This phenomenon was considered to be the most important in the influence of this drug on human immune functions. Circulating TNF- α induced by endotoxin in human volunteers was also significantly decreased [10]. Expression of IL-1 was suppressed by Ptx in cultures of human mononuclear cells in dose-dependent fashion [11]. Further studies provided evidence that Ptx differentially regulates the production of TNF- α and other cytokines. Meanwhile Ptx at high concentrations can bring about the production interleukin-6 (IL-6) by mononuclear cells but not other cytokines. When mononuclear cells were stimulated by lipopolysaccharide (LPS), Ptx was found to inhibit the secretion of TNF- α , in contrast, no inhibitory effect was observed on the induction of IL-6 [12]. Surprisingly, Ptx was able to modulate the effect interleukin-2 (IL-2) and production of soluble interleukin-2 receptor (sIL-2R). The IL-2 induced leukocyte-endothelial adherence was reduced by Ptx [13].

Table I

Inhibitory effects of pentoxifylline	Immune-endocrine interactions by TNF- α	
	Organs	Biological effects
+	brain	pituitary hormone regulation (fever)
+	endothelium	increase of leukocyte adhesion
+	immune effector cells	increase of NK and K cell activation
+	lymphocytes	enhancement of MHC and antigen
+	tissue cells	decrease of lipoprotein lipase

In the supernatant of lymphocyte cultures of healthy individuals induced by PHA an increased amount of sIL-2R was detected by monoclonal antibodies [14]. It

was observed that Ptx (1.0 mM) was able to inhibit IL-2 receptor expression as well as production of s IL-2R by 60% and 85%, respectively [15]. Summing up, effects of Ptx on cytokine network are responsible for improvement of treatment in endotoxic shock and adult respiratory distress syndrome (ARDS), tumour-associated symptoms, organ transplantation and other disorders. Here we will briefly summarize the major biological effects of TNF-alpha which has been known to be inhibited by Ptx (Table I).

Administration of Ptx in endotoxaemia and ARDS

TNF-alpha has been identified as an important mediator of lethal shock in animals [16]. Passive immunization against TNF-alpha attenuates the lethal effect of endotoxin in mice and protects against septic shock during Gram-negative bacteraemia in primates. Since Ptx has been shown to decrease the level of TNF-alpha, prospective studies were made for detecting its clinical effect. Zabel et al. [17] treated healthy volunteers with 100 ng endotoxin (*Salmonella abortus-equi*) and tested the level of cytokines. Both TNF-alpha and IL-6 were significantly increased 3 h after endotoxin injection. Three weeks later the volunteers were again treated with endotoxin and Ptx (500 mg) was also infused. There was no rise in TNF-alpha levels, though IL-6 levels rose in parallel with fever. In addition, administration of human recombinant TNF-alpha that is virtually endotoxin-free produces a constellation of metabolic derangements and lethal tissue injury that is almost identical to fetal endotoxic or septic shock and its deleterious effect can be abolished by Ptx. Further observations confirmed the beneficial influence of Ptx in endotoxic shock [18]. ARDS is characterized by increased metabolic activity of PMNs and enhanced pulmonary sequestration of leukocytes. The prospective studies supported the therapeutic effect of Ptx on ARDS. This result is in connection with inhibitory effect of Ptx on chemiluminescence activity of PMNs (Fig. 1).

Role of Ptx in neoplastic disorders

Cachectin was isolated from sera of patients with neoplastic disorders [19]. Amino acid sequencing of cachectin and cloning of the cDNA for the genes encoding cachectin and TNF-alpha, confirmed that the two molecules are identical [20]. Cachectin/TNF-alpha is a double-edged sword with both benefit (tumour necrosis activity) and negative attributes (cachectin activity). Ptx was used in patients with refractory metastatic lung cancer and found to improve symptoms after 4 days treatment. Ptx decreased the level of cachectin/TNF-alpha mRNA isolated from cancer patients and this was accompanied by a striking improvement in the quality of life. This observation suggest an inverse relation between cachectin/TNF-alpha and well-being [21]. IL-2 has been shown to induce the regression of metastatic cancer mediated by increased level TNF-alpha but clinical use has been limited due to associated toxicities. Therefore, it is of importance whether Ptx can influence toxic symptoms and survival. Four groups of C57BL/6 mice with pulmonary metastases from a methylcholantrene-induced fibrosarcoma and four groups of non-tumoured mice were treated with IL-2 alone, IL-2 and Ptx, Ptx alone or saline.

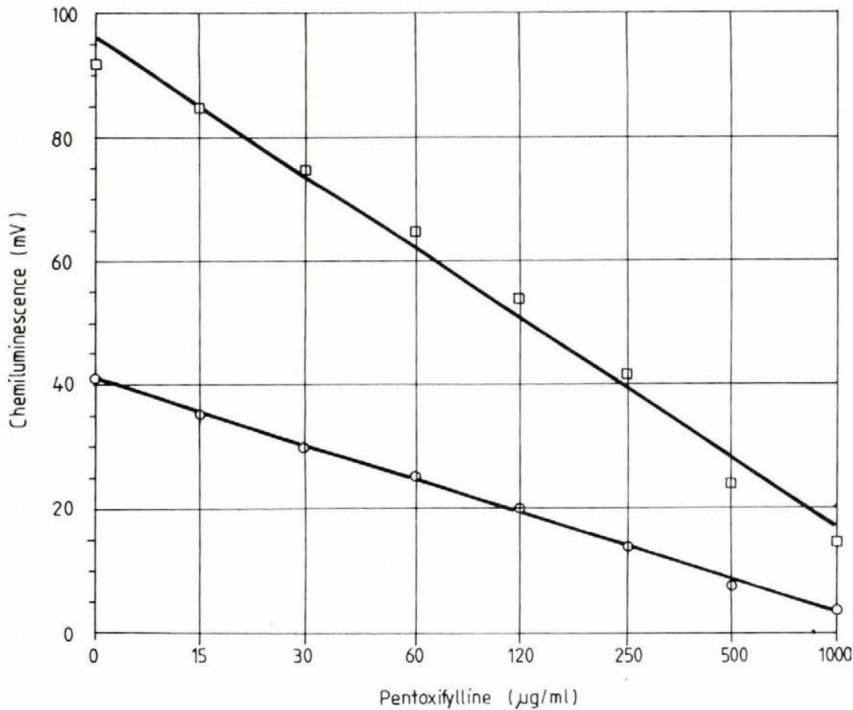


Fig. 1. Effect of Pentoxifylline on chemiluminescence activity of polymorphonuclear granulocytes induced by phytohaemagglutinin (PHA) and opsonized zymosan. Pentoxifylline decreased both the PHA (squares) and zymosan (open circles); induced fotonemission measured by LKB luminometer (LKB-1250)

It was found that Ptx abolished many of IL-2 induced effects including TNF- α production, lymphocyte infiltration and oedema of organs, hepatic dysfunction, leukopenia and thrombocytopenia as well. However, Ptx preserved antitumour efficacy while reducing morbidity and mortality caused by IL-2 treatment. Although in acute experiments Ptx has not been proved to assist the propagation of tumours, chronic administration might have a deleterious effect in patients without malignant disorders, since it can impair the natural killer cells (NK) activity [23]. The possible harmful effect of Ptx on NK cells requires further studies and precaution mostly in chronic administration.

Potential beneficial effects of Ptx in organ transplantation

In kidney transplanted patients anti-CD3 treatment used for prevention of graft rejection resulted in enhanced level of TNF- α and IL-2. In vitro experiments on human peripheral blood leukocytes indicated that Ptx alone or in synergy with methylprednisolone inhibited the release of TNF- α and IL-2 induced by anti-CD3

[24]. Phase I and II trials provided further evidence for beneficial effects of Ptx in prevention of transplantation related toxicities following bone marrow transplantation. In these patients Ptx was able to down-regulate the TNF-alpha production and reduce the graft-versus-host-disease (GVHD) [25].

Effect of Ptx on HIV infection

Increased TNF-alpha level has been observed not only in cancer patients but also in cachectic patients with AIDS. In addition, TNF-alpha is known to increase the expression of HIV-1 via activating its long terminal repeat. Moreover, TNF-alpha decreases the therapeutical efficacy of zidovudine (AZT). Significant decrease in HIV-1 replication by Ptx in infected human peripheral blood mononuclear cells is observed [26]. The preliminary data confirm that patients with AIDS may benefit from Ptx treatment because of its blockade of TNF-alpha mediated HIV-1 up-regulation and increased efficacy of AZT treatment [27].

Concluding remarks

There is a considerable body of evidence strongly implying that Ptx regulates the production of cytokines. Due to its significant inhibition on the synthesis and release of TNF-alpha acute administration of Ptx may be beneficial in pathological conditions characterized by the overproduction of TNF-alpha including septic shock, ARDS, inflammatory processes, prevention of GVHD in transplanted individuals. Influence of Ptx on well-being of patients with metastatic tumours is promising, however, it seems to be premature to draw the final conclusion. Since this drug might result in damage of NK cells activity, further experiments and observations are required to answer the important question of whether Ptx may increase the incidence of neoplastic disorders. Nevertheless, the chronic continuous administration of Ptx in peripheral arterial insufficiency is to be considered due to the potential enhanced susceptibility to malignant diseases.

Acknowledgement. This work was supported by the Ministry of Welfare, Hungary (Grant T-539/1990).

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GENOTYPIC AND PHENOTYPIC CHARACTERS AND NOSOCOMIAL SIGNIFICANCE OF BACTERIA ENDEMIC IN NEONATAL INTENSIVE CARE UNITS*

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The degree of colonization was determined by complex typing (sero-, phage-, colicin-, pyocin typing, plasmid profile analysis) of 212 *Escherichia coli*, 232 *Klebsiella*, 117 *Pseudomonas aeruginosa* and 52 *Staphylococcus aureus* strains isolated from nose, throat, ear and other sources of 563 new-born infants in gynaecological and maternity wards of two neonatal intensive care units (NICU I and II) during a one year period. The presence of *Klebsiella* strains was more frequent in NICU I and *E. coli* and *P. aeruginosa* in NICU II, *S. aureus* occurred in a low level in both units. In NICU I 34 kinds, in NICU II 43 kinds of *E. coli* serotype were found. In NICU I the accumulation of serotypes O6:H-, O6:H1, O19:H-, in NICU II O4:H-, O6:H1 was observed. The *Klebsiella* strains belonged in NICU I into 21, in NICU II into 12 phage types. *Klebsiella* was more frequent in NICU I than in NICU II, though the strains belonged to the same phage type in NICU II in 50.7%, but in NICU I 4 frequent and 19 rare phage types occurred. Sero- and pyocin typing was effective for typing of *P. aeruginosa*. The most frequent sero- and pyocin types were in NICU I: O11a,11b; 1h; in NICU II: O2a,2d,2f; 12v. The rate of antibiotic resistance in *E. coli*, *Klebsiella*, *P. aeruginosa* and *S. aureus* was nearly the same in both units, multiple resistance was more frequent in NICU I (except *P. aeruginosa*, it was multiple resistant in 100% in both units). In NICU I 267, in NICU II 174 infants were treated with antibiotics.

The administration of penicillin derivatives was nearly similar in the two care units and the resistance among *E. coli* and *Klebsiella* strains was nearly the same too. Though, cephalosporins were used more frequently in NICU II, resistance to cephalosporins among *E. coli* and *Klebsiella* was a bit higher in NICU I. Aminoglycosides were more often used in NICU I, resistance to aminoglycosides among *E. coli* and *Klebsiella* was higher in this unit. The rate of isolation of the examined bacteria was significantly lower in the group treated with antibiotics, than in the untreated group.

Infections caused by Gram-negative bacteria mainly *Escherichia coli*, *Klebsiella* and *Enterobacter* spp. are of the major hazards in neonatal intensive care units (NICU). It was proved by many publications, that the pathogens causing different diseases

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(pneumonia, septicaemia, meningitis, etc.) were derived from the bacterial flora of the infants [1-4]. The epidemiological connections of the infections are not well known probably due to the lack of effective typing methods for practical use. *E. coli* strains causing urinary tract infections in neonatal units were identified on the basis of their P-fimbria [5]. Nosocomial outbreaks caused by *Klebsiella* in neonatal wards were reported using phage typing [6, 7], capsule-swelling tests and R plasmid detection [8-10].

Long-term examinations were carried out in two neonatal intensive care units in a non-epidemic period using different typing methods to study the colonization and spread of *E. coli*, *Klebsiella* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa*. We wanted to know, if there were types which colonized more intensively within a bacterial species and which phenotypic and genotypic characters were connected with these types. It was also studied, whether the rate of colonization was influenced by the used antibiotic therapy.

Materials and methods

In two neonatal intensive care units of two maternity and gynaecological wards 1074 infants (in NICU I: 657, in NICU II: 417) were treated during the period between 1st October 1987 and 15th October 1988. Samples were obtained from 563 new-born infants (52.4%). A total of 2720 bacterial cultures was examined. Samples were taken from the infants from the nose, throat, ear and other sources at admission and leaving. Complex typing of the isolated *E. coli*, *Klebsiella* *P. aeruginosa* and *S. aureus* was carried out.

Complex typing of E. coli.

Serotyping. Determination of O and H antigen was carried out according to Ørskov and Ørskov [11], modified as described previously [12].

Determination of antigen K was carried out using specific phages K1 [13], K5 [14], K2, K7, K9, K12, K13, K20, K27, K30, K31, K36, K42 [15].

Phage typing. The method described by Milch and Gyenes [16] and Milch [17] was used.

Testing for colicin production and colicin typing was carried out according to Milch and Gyenes [16] and Lafont et al. [18].

Aerobactin production (Aer) was determined by qualitative biologic assay described by Carbonetti and Williams [19].

Haemolysin production (Hly) was examined according to Cowan and Steel [20].

Sensitivity to antibiotics was tested using "Resistest" discs (Human Institute for Serobacteriological Production and Research, Hungary), ampicillin (AM), carbenicillin (CB), azlocillin (AZL), mezlocillin (MZ), cefalotin (CF), cephalixin (CX), cefamandole (MA), cefuroxime (CXM), ceftriaxone (CTR), cefotaxime (CTX), tetracycline (TE), chloramphenicol (C), streptomycin (S), kanamycin (K), neomycin (N), gentamicin (GM), tobramycin (TM), netilmicin (NET), amikacin (AN), sulfamethoxazole trimethoprim (SXT), nitrofurantoin (FT), nalidixic acid (NA).

Plasmid profile analysis. Plasmid DNA was prepared according to Kado and Liu [21].

Agarose gel electrophoresis was performed in vertical agarose gels according to Meyers et al. [22].

Plasmid standards used to determine molecular weight. *E. coli* V517 [23]: 1.4, 1.8, 2.0, 2.6, 3.4, 3.7, 4.8, 35.8 Md, R1:62 Md, R27:112 Md, pSp1:140 Md.

Complex typing of Klebsiella.

Phage typing was carried out according to Slopek et al. [24].

Sensitivity to antibiotics and plasmid profiles were determined as described in E. coli complex typing.

Phage typing of Staphylococcus aureus was carried out according to the Report of International Subcommittee on Phage Typing of Staphylococci [25] completed with newly isolated typing phages.

Sensitivity to antibiotics was tested using besides the above mentioned antibiotics the following disks: penicillin (PV), oxacillin (OX), oleandomycin (OL), clindamycin (CM), lincomycin (L), spectinomycin (SPT).

To determine subspecies Meyer's method [26] was used.

Complex typing of Pseudomonas aeruginosa.

Phage typing was carried out according to Lindberg and Latta [27].

Pyocin typing. The method of Gillies and Govan [28] was used.

Sensitivity to antibiotics and plasmid profile analysis was determined as described in Complex typing of *E. coli*.

Antibiotic agents used for therapy: penicillin (Biogal), oxacillin, methicillin (Chinoin), azlocillin (Securopen, Bayer), mezlocillin (Baypen, Bayer), ampicillin (Penbritin, Beecham), cefuroxime (Zinnat, Zinacef, Glaxo), cephalixin (Pyassan, Chinoin), cefoperazone (Cefobid, Pfizer), cefamandole (Mandokel, Lilly), cefotaxime (Claforan, Roussel-Uclaf), cefoxitin (Mefoxin, Merck Sharp), ceftriaxon (Rocephin, Hoffmann La Roche), gentamicin (Chinoin), tobramycin (Brulamycin, Biogal), amikacin (Amikin, Bristol), netilmicin (Netromicine, Schering), nitrofurantoin (Chinoin), sulphamethoxazole-thrimethoprim (Sumetrolim, Egis).

For statistical analysis $n \times n$ contingency table, for significance the chi-square test was used [29].

Results

Out of the examined 2720 samples (NICU I: 487, NICU II: 364) opportunistic pathogens were identified from 851 samples (31.3%); out of the 1019 isolated cultures 613 were examined.

Escherichia coli

Table I shows the origin and source of the isolated *E. coli* strains. (Complex typing of 212 *E. coli* strains (81 from NICU I and 131 from NICU II) was carried out.)

Results of serotyping: Distribution of serogroups according to origin is demonstrated in Table II. Among the strains isolated in the NICU I 15, in NICU II 24 serogroups were found; not typable (Nt) and spontaneously agglutinating (Sp) strains were also found.

The most frequent serogroups, found in NICU I were O4 (14) O6 (19) and O75 (6) (Table II). The majority of strains of serogroups O4 (9) belonged to serotype O4:H1. Among the strains of serogroup O6 the most common serotypes were O6:H- (7) and O6:H1 (10). The presence of O19:H- (4) and O75:H7 (4) were also frequent (Table II). Examining the distribution of serogroups according to the source of isolation it was found, that serotype O4:H1 occurred most frequently in nose samples and serotype O6:H1 in throat samples (Table III).

In NICU II the following serogroups were the most frequent: O1 (11), O2 (9), O4 (22), O6 (21), O18 (10) and O21 (7); the most frequent serotypes were O1:H- (7), O4:H5 (12), O6:H1 (20), O18:H- (7), O21:H- (6) (Table II). Serotype distribution according to sources is shown in Table III. The same serotype (O6:H1) was the most frequent among throat samples, as in NICU I and this serotype was frequently isolated also from ear samples.

Table I

Origin of E. coli strains examined by complex typing

Source of isolation	Origin	
	NICU I	NICU II
Nose	12	23
Throat	39	44
Ear	13	36
Urine	3	10
Cervix	1	13
Abdominal cavity	1	—
Bile	1	—
Blood	—	1
Skin	—	1
Wound	—	1
Other	11	2
Total No. of examined strains	81	131

Distribution of frequent serotypes according to phage patterns: NICU I. The 9 strains of serotype O4:H1 were isolated from 5 infants between June 1987 and July 1988, 3 kinds of phage patterns were found. The 10 strains of serotype O6:H1 were isolated from 9 infants and one mother, between November 1987 and June 1988, they belonged to 4 phage patterns, strains isolated from 5 infants belonged to the same phage pattern (4a, 4b, 13, 23). The 7 strains of serotype O6:H— were derived from 6 infants between January 1988 and July 1988, they belonged to 2 phage patterns. Correlation was found among the strains of serotype O6:H— on the basis of their phage patterns in NICU I, strains of the same phage pattern were isolated from one ear- and 3 nose- and throat samples of 2 infants. Serotype O19:H—: strains of the same phage pattern were isolated from ear-, throat-, wound samples of 4 infants, among strains of serotype O75:H7 the same phage pattern was found in the throat samples of 4 infants.

NICU II. Serotype O1:H—: the 7 strains isolated from 4 infants and one mother between October 1987 and June 1988 belonged to 3 phage patterns. Serotype O4:H—: the 6 strains isolated from 2 infants and 1 environmental sample during the period June to November 1987, were nearly the same in phage pattern. Serotype O4:H5: the 12 strains isolated from 5 infants between June and November 1987 belonged to 3 phage patterns, Serotype O6:H1: the 20 strains isolated from 14 infants and one mother between June 1987 and October 1988 belonged to two phage patterns. The four strains belonging to phage pattern 4a, 4b, 13, 23 were isolated from throat- and ear samples of 3 infants, the 8 strains of phage pattern 4a, 4b were isolated from nose-, throat-, ear-, wound- and urine samples of 7 infants and one mother. Serotype O18ac:H—: the 7 strains derived from 4 infants (isolated between June 1987 and September 1988) belonged to 4 phage patterns.

Table II

Distribution of E. coli isolates according to serotypes and serogroups

Serotype	NICU I		Serotype	NICU II	
	No. of			No. of	
	serotypes	serogroups		serotypes	serogroups
O :H-	1		O :H-	7	
:H5	1	2	:H7	4	11
O :H-	1		O :H-	5	
:H1	9		:H1	2	
:H5	3		:H42	1	
:HNt	1	14	:HNt	1	9
O :H-	7		O :H-	6	
:H1	10		:H1	1	
:H5	1		:H4	1	
:HNt	1	19	:H5	12	
O :H-	3		:HNt	2	22
:H4	1	4	O :H4	1	1
O1 :H2	1	1	O :H1	20	
O1 :H7	3		:HNt	1	21
:H55	1	4	O :HNt	1	1
O1 :H-	4	4	O :HNt	1	1
O2 :H1	2	2	O1 :H32	2	
O4 :H55	2	2	:HNt	1	3
O7 :H18	2	2	O1 :HNt	1	1
O7 :H-	1		O1 :H-	7	
:H7	4		:H7	2	
:HNt	1	6	:H14	1	10
O8 :H-	2	2	O1 :H-	2	2
O10 :HNt	3	3	O2 :H-	6	
O11 :H4	1	1	:HNt	1	7
O13 :H-	1	1	O2 :HNt	1	1
OS :H-	1		O3 :HNt	1	1
:HNt	3	4	O3 :HNt	1	1
ON :H-	3		O4 :H7	1	1
:H9	1		O4 :H4	1	1
:H12	1		O7 :H7	1	1
:H26	1		O7 :HNt	1	1
:H40	1		O8 :H12	1	1
:HNt	3	10	O11 :H16	1	1
			O12 :H1	1	1
			O14 :H34	1	1
			O15 :HNt	1	1
			OS :H-	3	
			:HNt	2	5
			ON :H-	9	
			:H4	3	
			:H16	1	
			:HNt	12	25
Total	81	81		131	131

Table III

Distribution of the most common E. coli serotypes according to the source of isolation (NICU I and NICU II)

Origin	Source	Total No. of <i>E. coli</i>	Most common serotypes
NICU I	Nose	12	O4:H1 (5)
	Throat	39	O4:H1 (4), O6:H1 (8), O75:H7 (4)
	Ear	13	O6:H- (2), O19:H- (2)
NICU II	Nose	23	O4:H5 (6)
	Throat	44	O4:H5 (4), O6:H1 (9), O18:H- (4)
	Ear	36	O4:H- (3), O4:H5 (4), O6:H1 (9)
	Cervix	13	ONt:Hnt (4)
	Urine	10	O12:H32 (2)

The 12 ONt:Hnt strains were isolated from 6 infants and 2 mothers between July 1987 and October 1988 belonged to 7 kinds of phage patterns. The 9 ONt:H- strains were isolated from 8 infants and one mother between March 1987 and October 1988 and they belonged to 6 phage patterns. *NICU II*. Strains of serotype O1:H- and belonging to the same phage pattern were isolated from the cervix of a mother and the throat and ear of her infant. Strains of serotype O4:H- belonging to the same phage pattern were derived from the nose and ear of an infant and the incubation tube. Strains of serotype O4:H5, of the same phage pattern were found in the nose, throat and ear samples of 3 infants. Among the strains of serotype O6:H1 those of phage pattern 4a, 4b were found in ear samples of 4 infants, in the urine of 1 infant, in 6 throat samples and in the cervix sample of a mother. The strains of the same serotype and characterized by phage pattern 4a, 4b, 13, 23 were isolated also from ear and throat samples.

Table IV

Plasmid profile analysis of 86 E. coli strains

Plasmid profile	NICU I			Plasmid profile	NICU II		
	Serotype		No. of examined strains		Serotype		No. of examined strains
	Determined	Nt			Determined	Nt	
Determined	18	8	26	Determined	44	16	60
plasmid profile	15	5	20	plasmid profile	31	16	47
Plasmid-free	3	3	6	Plasmid-free	13	—	13

Table V
Plasmid profiles of E. coli serotypes

NICU	Serotype	Plasmid profile	Phage pattern	No. of strains	Total No. of	
					plasmid profiles	phage patterns
I	O6: H1	Plasmid free	4a 4b	2		
	(5)*	20.5 5.5	4a 4b 13 23	2	2	2
		12 5.8 2.5	4a 4b 13	1		
	O107:Hnt	7.7 6.5 3.8 3.3	Nt	3	1	1
II	O4:H5	Plasmid free	4a 4b 16 17 20	4		
	(7)		4a 4b; 4a 20		3	3
		85	4a 4b	1		
		94 26.5	4a 4b	2		
	O6:H1	20.5 5.5	4a 4b 13 23	1		
	(7)	20.5 (10) 5.3	4a 4b 13	1		
		71 28 14.5	4a 4b 13	1		
		71 2.3	4a 4b 13	2	5	1
		5.5 2.2	4a 4b 13 (16)	1		
		22 9.0 4.3 21	4a 4b 13 23	1		
	O18ac:H-	74 50 2.0	4a 4b 17 K5	1		
	(5)	68 3.8 3.7 2.5 15	12 14	2	3	4
		75 53	16 17 K5	1		
		12 5.8 2.2	4a 4b 13	1		
	ONt:H-	120 54 44 1.2	12 13 14	1		
	(6)	32 17.5 5.3 4.7 4.3 3.8	15 23 K1	1		
		3.4 1.6 1.4 1.2				
		88 4.9 1.5	Nt	1	6	4
		90 5.4 3.7 3.5 3.3 1.4 1.2	Nt	1		
		80 5.5 4.4 3.1 1.1	2 3 6 7	1		
		95 70 20 16.5 5.3	Nt	1		
		3.1 2.8 2.4 1				

* No. of examined strains

Plasmid profiles within serotypes. Plasmid profile of 26 *E. coli* strains originated from NICU I was examined, 18 strains had determined serotype and 8 strains were serologically not typable. Plasmid profile could be determined from 20 strains, 15 serologically typable and 5 serologically not typable, plasmid-free were 3-3 strains. Plasmid profile of 60 strains were examined from NICU II, 44 isolates were serologically typable and 16 were serologically not typable. Plasmid profile could be determined from 31 strains, 13 were plasmid-free from the first group and they were all (16) determinable from the second group (Table IV). The number of plasmid profiles

corresponded to the number of phage patterns within a serotype in 3 cases and it did not in 3 cases (Table V).

Aerobactin production as a virulence factor was examined in 212 strains (from NICU I and II). Aerobactin was produced by 76 strains (35.8%), the ratio being similar in both units (37.0 and 35.1%). Among the most common serotypes aerobactin producers were found in serotypes O4:H1, O6:H1, O6:H- in NICU I and O1:H7, O4:H-, O6:H1, O18:H-, O21:H- in NICU II (Table VI).

Occurrence of antigens K1, K5. In NICU I K1 carrying 12 *E. coli* strains were isolated from throat-, ear samples of 16 infants and from the vagina of one mother (14.8%). The strains isolated from the vagina of the mother and from the ear and throat samples of her infant belonged to the same sero- and phage pattern (O1:H-, 23). In NICU II, K1 antigen containing 15 *E. coli* strains (11.4%) were isolated from nose-, throat, ear- and urine samples of 11 infants. K5 antigen containing strains were isolated from throat samples of 4 infants (4.9%) in NICU I, and 8 strains (6.1%) from throat-, ear and urine samples of 6 infants and from the vagina of 2 mothers in NICU II (Table VII).

Table VI

Occurrence of aerobactin producing E. coli strains in NICU I and II

NICU I			NICU II			NICU I + NICU II		
Aer + strains		Total No.	Aer + strains		Total No.	Aer + strains		Total No.
Frequent serotypes		of <i>E. coli</i>	Frequent serotypes		of <i>E. coli</i>	Frequent serotypes		of <i>E. coli</i>
No.	%	strains	No.	%	strains	No.	%	strains
30	37.0	O4:H1, O6:H1	46	35.1	O6:H1, O4:H-	76	35.8	212

Table VII

Occurrence of K1 and K5 antigen containing E. coli strains in NICU I and NICU II

Antigen	NICU I			NICU II		
	Strains		K antigen	Strains		K antigen
	No.	%*	positive serotypes	No.	%*	positive serotypes
K1	12	14.8	O2:H5, O7:H-, O18ac:H7, ONt:H-	15	11.4	O1:H-, O1:H7, O18ac:H7, O45:H7, OSp:HNt
K5	4	4.9	O6:H-, O6:H1	8	6.1	O6:H1, O6:HNt, O18ac:H-, OSp:H-

* Referred to the total No. of *E. coli* strains

Klebsiella

During the period of examination in *NICU I*, 165 *Klebsiella* strains were isolated from 102 infants and 3 environmental samples; the 165 strains belonged into 21 phage types and 9 strains were not typable. *Klebsiella* was isolated from the different samples in 2 to 5 times from 42 infants; they belonged into the same phage type in case of 27 infants.

Table VIII

Phage type distribution of Klebsiella strains in NICU I and II

Phage type	No. of strains	
	NICU I	NICU II
I.A1	6	6
I.A2	1	1
I.A8	2	—
I.A13	—	1
I.A21	3	—
I.A22	27	—
I.B1	16	2
I.B5	5	—
I.B18	1	—
I.B19	9	—
I.B26	1	—
I.B30	1	—
I.C12	1	—
II.A1	16	34
II.B2	3	—
III.B6	—	2
III.B7	—	1
IV.A1	1	3
IV.A2	—	1
IX.A2	—	1
X.A4	2	—
XI.A1	8	—
XI.A2	45	1
XIII.A1	1	—
XV.A1	1	—
K1 4	6	4
Nt	8	9
C14*	1	1
Total	165	67

* *E. cloacae* strain

Table IX

Phage type distribution of Klebsiella strains according to the source of isolation

Phage type	Source of isolation															
	Nose		Throat		Ear		Urine		Blood		Environment		Other***		Total	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
IA1	2	–	3	4	3	–	–	1	–	–	–	–	–	–	8	5
IA22	12	–	10	–	5	–	–	–	–	–	–	–	–	–	27	–
IB1	4	–	9	–	2	1	–	1	–	–	–	–	1	–	16	2
IB5	1	–	4	–	–	–	–	–	–	–	–	–	–	–	5	–
IB19	5	–	6	–	1	–	–	–	–	–	–	–	–	–	12	–
IIA1	5	12	6	13	3	7	–	–	–	–	–	–	1	1	15	33
XIA1	1	–	5	–	–	–	–	–	–	–	–	–	–	–	6	–
XIA2	18	–	21	–	5	1	–	–	–	–	1	–	1	–	46	1
K14*	1	–	2	1	1	1	–	–	–	–	–	–	–	1	4	3
Nt	1	1	3	2	3	4	–	1	–	–	–	–	–	1	7	9
Other**	3	5	11	2	3	5	–	1	–	1	2	–	–	–	19	14
Total	53	18	80	22	26	19	–	4	–	1	3	1	3	2	165	67

Source of isolation: I = NICU I, II = NICU II

* Lysed by additional phage

** IA2, IA8, IA13, IA21, IB18, IB26, IB30, IC12, IIA11, IIB2, IIB6, IIB7, IVA1, IVA2, VIIA3, IXA2, XA4, XIIA1, XVA1, C14

*** Navel, eye, intubator, vulva

Table X

Incidence of Klebsiella plasmid profiles according to phage types in NICU I

Phage type	Plasmid profile (Md)	No. of plasmid profiles	No. of strains
I.A1	62 36	3-kinds	1
	112 3 2.6 2.2 1.4		1
	112		2
I.A8	112	2-kinds	1
	112 1.6		1
I.A22	112	1-kind	5
I.B1	112 5.6	4-kinds	1
	140 112 3.5		1
	112 3.4		1
	112		1
	Plasmid free		1
I.B5	140 112 3.6	1-kind	2
I.B18	112	1-kind	1
I.B19	112 60	2-kinds	1
	112		3
I.B30	Plasmid free		1
II.A1	112 60	7-kinds	1
	112 3.4 2.2		1
	112 2.6		2
	112 60 2.6		1
	68 2.6		1
	112 62 3.2		1
	140 112 70		1
IV.A1	36	1-kind	1
XI.A1	140 112 70 3.5	4-kinds	4
	70		1
	70 5.1		1
	112		1
XI.A2	140 112 3.6	6-kinds	3
	140 112		1
	112 3.0		3
	112		4
	112 5.6		4
	140 112 4.6		1
	Plasmid free		2
Nt	112 3.7 2.4 2.2 1.4	4-kinds	1
	68		1
	112 60 2.4 2.2		1
	107 76		1
K14	62	2-kinds	1
	140 112 3.6		2
No. of examined strains			65

In NICU II, 67 *Klebsiella* strains were isolated from 49 infants and one object, 57 strains belonged into 12 phage types and 9 were not typable. *Klebsiella* was isolated from the different samples in 2 to 5 times from 13 infants, they belonged to the same phage type in case of 8 infants.

Table VIII shows the distribution of *Klebsiella* phage types in NICU I and II, phage type distribution according to the origin of the strains is demonstrated in Table IX. Phage types of strains isolated from different environmental specimens and rinsing fluids were I.B1, II.A1, XI.A2, C14 (*Enterobacter cloacae*) in NICU I and Nt in NICU II. The frequent phage types are shown in chronological order in Figs 1 and 2. These are in NICU I: XI.A2, I.A22, I.B1, II.A1, I.B19, XI.A1, Nt; in NICU II: II.A1, Nt, I.A1. The phage types occurring in both units were: I.A1, I.A2, I.B1, II.A1, IV.A1, XI.A2, K14, Nt, C14.

Table XI

Incidence of Klebsiella plasmid profiles according to phage types in NICU II

Phage type	Plasmid profile (Md)	No. of plasmid profiles	No. of strains
I.A1	36	4-kinds	2
	70 2.6		1
	112 70		1
	112 62 3.4		1
I.A2	112	1-kind	1
II.A1	112 60 2.6	5-kinds	1
	2.2		1
	140 112 3.5		1
	140 112 70 3.5		1
	140 112		1
III.B6	70 2.6	1-kind	1
III.B7	70	1-kind	1
IV.A1	70 62	2-kinds	1
	80 62		1
IV.A2	70 3.4	1-kind	1
K14	112 3.4 2.2	2-kinds	1
	112 60 2.4 2.2		1
	112 62 2.2 1.9		1
Nt	70	5-kinds	2
	36 2.2		1
	70 50		1
	36		1
	112 60 2.4 2.2		1
	Plasmid free		1
<i>Enterobacter</i>	112 62 3.4		1
No. of examined strains			27

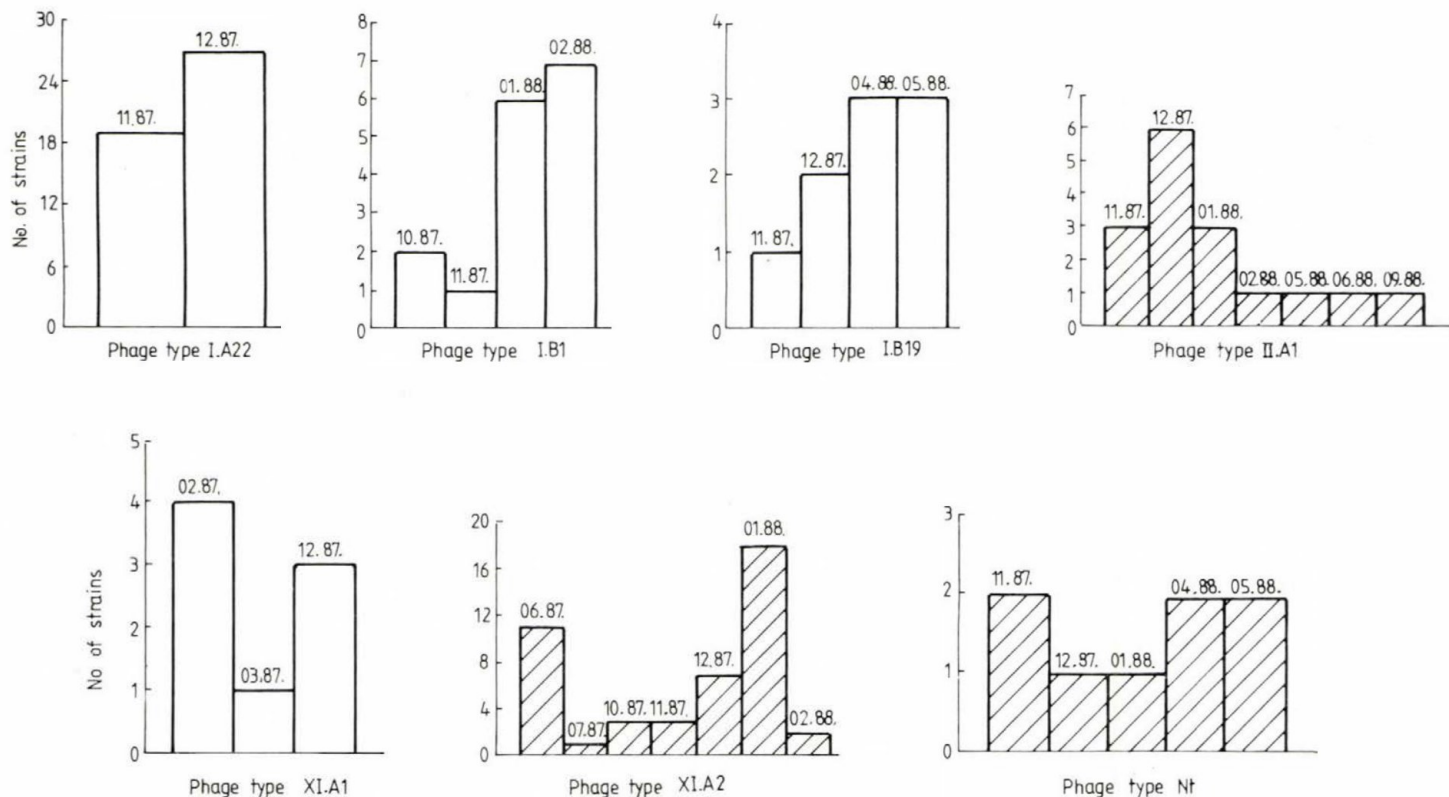


Fig. 1. Sequential occurrence of the frequent *Klebsiella* phage types in NICU I. Open columns: short period spread of phage type IA22, IB1, IB19, XI.A1. Shaded columns: long period spread of phage type IIA1, XI.A2, Nt

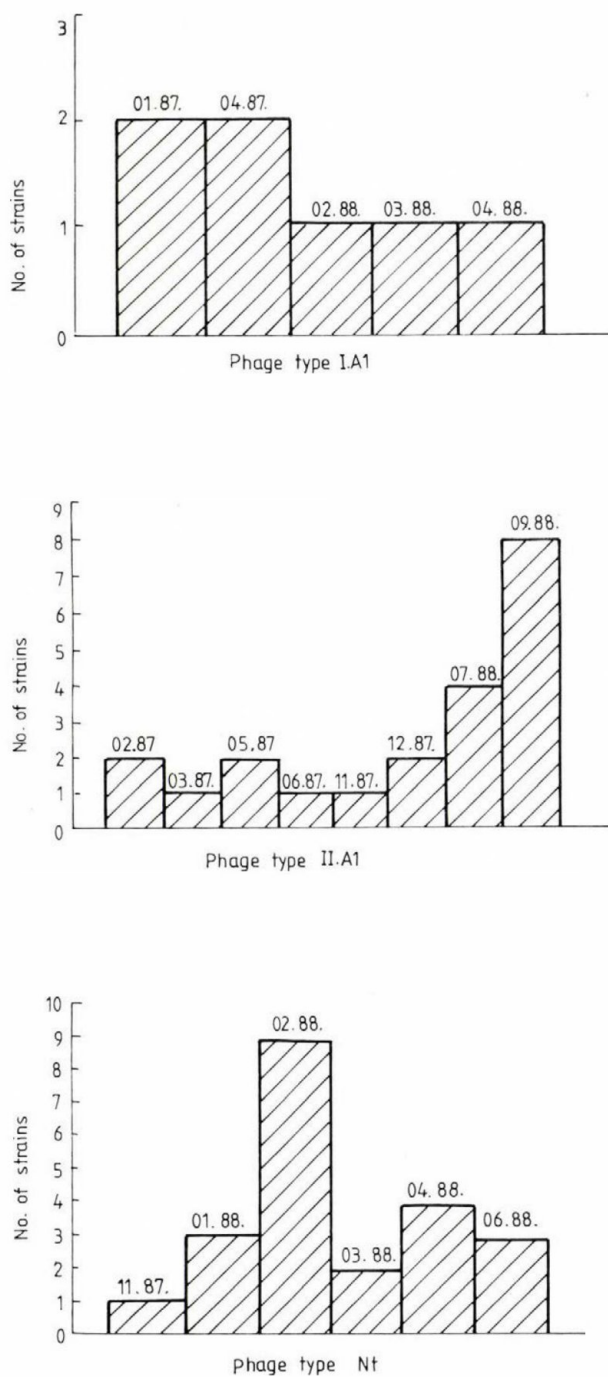


Fig. 2. Sequential occurrence of the frequent *Klebsiella* phage types in NICU II. Shaded columns: long period spread of phage type I.A1, II.A1, Nt

Plasmid profile examinations on Klebsiella strains. NICU I. The number of examined strains was 65, 4 strains were plasmid free. Table X shows the plasmid profiles according to phage types. Frequent plasmid profiles were: 140, 112 (70) (3.5); 112. *NICU II.* The number of examined strains was 27, 1 strain was plasmid free. Table XI shows the plasmid profiles according to phage types. Frequent plasmid profiles were: 112, 60, 3.4; 70, 3.4.

Pseudomonas aeruginosa

In *NICU I* during the period of examination 62 strains were isolated from nose, throat, ear, trachea and eye samples of infants and from the inhaling rinsing fluid of the perfusor, vaporizing tank and its fluid. The strains belonged to 4 serogroups or were serologically not typable; 23 phage types and 27 pyocin types were found. In repeated isolations the same serogroup was found in 12 persons, different serogroups in one person, the same phage- and pyocin type in 11 persons; different phage types did not occur in samples of the same person, different pyocin types were found in 3 persons. Fifty-five strains belonged to 9 kinds of plasmid profiles, 36 strains were plasmid free (Table XII).

Table XII

Frequent serogroups phage- and pyocin types and plasmid profiles of P. aeruginosa

Origin	Serogroup ⁺⁺	Phage types	Pyocin	Plasmid profile (Md)
NICU I	O11a, 11b (46)*	2/21/44/68/F8/	1h (20)	44 (14)
	Other (16)	1214/M6/C18/C21 (15)	Other (42)	Other (9)
		1214/C18 (5)		Plasmid free (37)
		Other (42)		
NICU II	O2a, 2d, 2f (19)	16/44/109/1214 (10)	12v (10)	60 69 (7)
	Other (36)	Other (45)	Other (45)	Other (7)
				Plasmid free (20)

* Bracketed figures: No. of strains

⁺⁺ According to B. Lányi, T. Bergan 1978. Serological characterization of *Pseudomonas aeruginosa*, p93-168. In T. Bergan and J. Norris (ed.), *Methods in Microbiology*, vol. 10. Academic Press, Inc., New York

In *NICU II* 55 strains were isolated from nose, throat, ear, trachea, wound and blood samples and from one environmental specimen. The strains belonged to 6 serogroups or were not typable, and to 26 phage and pyocin types. The same serogroup occurred in 7 infants, different serogroups in one person; 5 infants carried the same phage- and pyocin type and different phage- and pyocin types were found in one infant. Forty strains were divided into 9 plasmid profile types, 20 strains were plasmid free. In *NICU I* the most frequent complex type was serogroup O11a, 11b; phage type 2/21/44/68F8/1214/M6/C18/C4/14; pyocin type: 1h; plasmid free. In *NICU II* the most frequent complex type was serogroup O2a, 2d, 2f; phage type: 16/44/109/1214; pyocin type: 12v; plasmid profile: 69 Md.

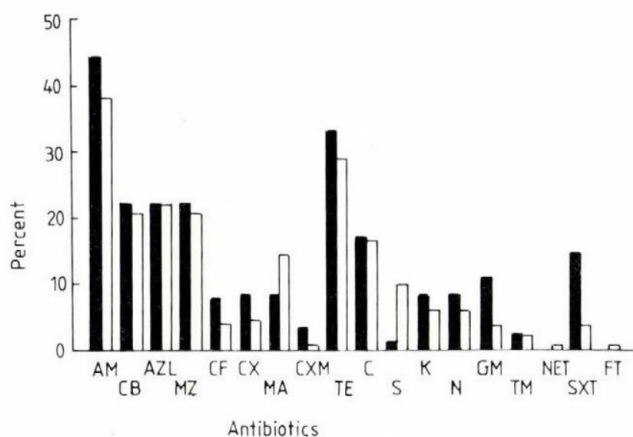


Fig. 3. Antibiotic resistance of *E. coli*. Solid columns: NICU I ; hatched columns: NICU II

Table XIII

Incidence of E. coli, Klebsiella, P. aeruginosa and S. aureus strains in NICU I and II

		No. of isolated strains	Positive newborns No.	%	Total No. of examined persons
NICU I	<i>E. coli</i>	81	59 ^a	16.5	357
	<i>Klebsiella</i>	165	110	30.8	
	<i>P. aeruginosa</i>	62	45 ^b	12.6	
	<i>S. aureus</i>	27	25	7.0	
	Total	335			
NICU II	<i>E. coli</i>	131	89 ^c	43.2	206
	<i>Klebsiella</i>	67	52	25.2	
	<i>P. aeruginosa</i>	55	42 ^d	20.4	
	<i>S. aureus</i>	25	15	7.2	
	Total	278			
Total	<i>E. coli</i>	212	148	26.3	563
	<i>Klebsiella</i>	232	162	28.8	
	<i>P. aeruginosa</i>	117	87	15.5	
	<i>S. aureus</i>	52	40	7.1	
	Total	613			

^a Including one mother

^b Including 8 environmental specimens

^c Including 8 mothers

^d Including one environmental specimen

Staphylococcus aureus

NICU I: the 27 strains were derived from 25 infants. Nineteen phage patterns were found. Frequent phage pattern was not found (94/96 V and 53 III occurred twice). The same phage pattern was found from different samples of the same person in case of 2 infants.

NICU II: the 25 strains were derived from 15 infants, 14 phage patterns were found, the most common phage pattern was: 84 III (7 strains). The same phage pattern from different samples of the same person was found in case of 4 infants, different phage pattern in case of one infant.

Table XIII summarizes the incidence of *E. coli*, *Klebsiella*, *P. aeruginosa* and *S. aureus* strains. In NICU I, out of 357 infants 81 *E. coli* strains were isolated from 58 infants and one mother (*E. coli* was carried by 16.5% of the infants). In NICU II, *E. coli* was isolated out of 206 infants from 81 and from 8 mothers (43.2%). *Klebsiella* spp. were isolated in 30.8 and 25.2% from the infants in NICU I and II, respectively. *P. aeruginosa* was isolated from the infants in 12.6 and 20.4% in NICU I and II. *S. aureus* was demonstrated in 7.0 and 7.2% in NICU I and II. It was observed, that in NICU II nearly half of the infants (43.2%) were colonized by *E. coli* and 1/5 (20.4%) by *P. aeruginosa*. *Klebsiella* occurred more frequently in NICU I than in II, but according to phage types the strains belonged to the same phage type in 50.7% in NICU II, but in NICU I they belonged to 4 frequent types (Table XIII).

Sensitivity to antibiotics

E. coli strains resistant to antibiotics occurred in nearly the same percentage in both units, multiple resistance (resistant to more than three antibiotics) was more frequent in NICU I (Table XIV). Figure 3 shows the antibiotic resistance of *E. coli* strains in percentages derived from the two units. Resistance to AM, CX, CXM, TE, GM, K, N, SXT occurred in higher percentages in NICU I, resistance to MA and S was more frequent in NICU II. Resistance to CB, AZL, MZ, C and TM was almost similar in the two units, resistance to CTR, CTX, AN, NA was not found in either units.

Resistance among *Klebsiella* strains was higher in 3% in NICU I and the multiple resistance was higher in 30%, than in NICU II. Figure 4 shows the antibiotic resistance of *Klebsiella* strains in percentages derived from the two units. In NICU I, resistance was more frequent to AZL, MZ, CX, MA, CXM, TE, C, GM, TM, NET, SXT; in NICU II, S, K, N. Resistance to AM, CB, CF was nearly the same in both units.

P. aeruginosa strains were resistant in 100% in both units and all were multiple resistant (Table XIV). Figure 5 shows the antibiotic resistance of *P. aeruginosa* strains in percentages derived from the two units. Resistance was more frequent to AM, CB, AZL, MZ, CF, CX, CTR, S in NICU I, to CXM, K and SXT in NICU II. Resistance was nearly the same to MA, TE, C, N, GM, FT, NA in both units, resistance to TM, NET and AN was not observed in either unit.

Table XIV

Sensitivity to antibiotics of E. coli, Klebsiella, P. aeruginosa and S. aureus strains isolated in NICU I and II

NICU	Species	No. of examined strains	Sensitive	Percentage of strains	
				resistant	multiple resistant
I	<i>E. coli</i>	81	44.4	55.6	34.6
II		131	46.6	53.4	24.4
I	<i>Klebsiella</i>	165	—	100.0	95.6
II	spp.	67	3.0	97.0	64.2
I	<i>P. aeruginosa</i>	62	—	100.0	100.0
II		55	—	100.0	100.0
I	<i>S. aureus</i>	27	7.4	92.5	81.4
II		25	—	100.0	76.0

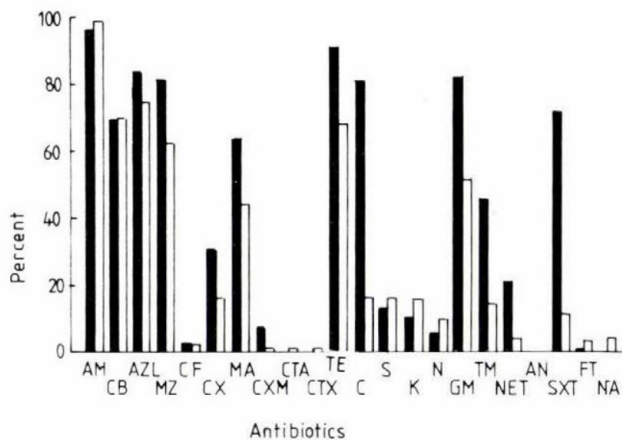


Fig. 4. Antibiotic resistance of *Klebsiella*. Solid columns: NICU I; hatched columns: NICU II

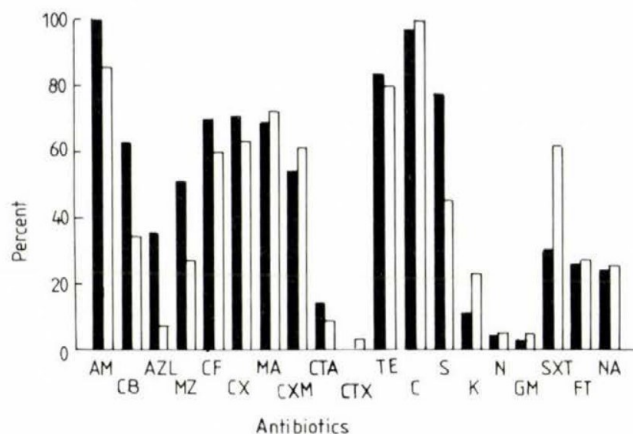


Fig. 5. Antibiotic resistance of *P. aeruginosa*. Solid columns: NICU I; hatched columns: NICU II

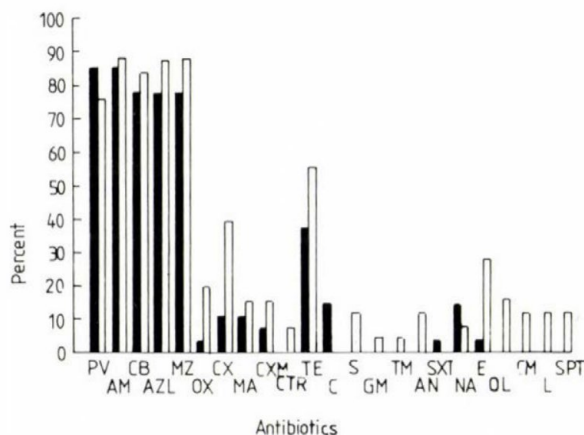


Fig. 6. Antibiotic resistance of *S. aureus*. Solid columns: NICU I; hatched columns: NICU II

Although resistant *S. aureus* strains were found in NICU II in a bit higher percentage than in NICU I the rate of multiple resistant strains in NICU I was higher (Table XIV). Figure 6 shows the incidence of antibiotic resistance of *S. aureus* strains isolated from the two units, in percentages. Resistance to PV, C, NA was more frequent in NICU I, to AM, CB, AZL, MZ, OX, CX, MA, CXM, CTR, TE, S, GM, TM, AN, E, OL, CM, L, SPT in NICU II, resistance to CTX, K, N, NET and FT was not observed in either unit.

Discussion

In two neonatal intensive care units nose, throat and ear samples were taken from 563 infants and 9 mothers during a one-year period and 212 *E. coli*, 232 *Klebsiella* (and *E. cloacae*), 117 *P. aeruginosa* and 52 *S. aureus* strains were isolated. The study aimed to determine the measure of colonization and spread of the above listed bacteria. Sprint et al. [30] demonstrated, that in 15% among new-borns from whose nose and throat other bacteria were isolated, than haemolytic streptococci, the following diseases were caused mainly by the colonized bacterial flora. Other authors [31–33] found no connection between colonization and the subsequent sepsis or meningitis. These contradictions can be explained by the lack of simple and effective typing methods. The identification of Gram-negative opportunistic pathogens is usually carried out only in case of epidemics.

Examinations on the spread of infections caused by *E. coli* were carried out using serotyping [34] and/or phage typing [16, 17], presence of *E. coli* in NICU-s was not studied by complex typing. New-borns are infected also by *Klebsiella* spp., besides *E. coli* [35]. The epidemiological studies were also inhibited by the lack of typing methods easy to accomplish. To make clear these questions we used the available complex typing methods for characterization of *E. coli*, *Klebsiella*, *P. aeruginosa* and *S. aureus* strains isolated from nose, throat and ear samples of infants and from their environment in two NICU-s.

Out of the frequently occurring *E. coli* serogroups (O1, O4, O6, O18, O21, O75) in NICU I and II, it was found in our previous examinations carried out on 3334 strains belonging to serogroups O4, O6, O18, that certain pathogenic markers (Hly and MRHA) were frequent in these serogroups [36]. According to publications [37] and our findings [36, 38], K1 antigen carrying *E. coli* strains have a significant role in neonatal meningitis. *E. coli* strains possessing antigen K1 was found in 14.8 and 11.4% in NICU I and II, respectively. The occurrence of antigen K5 carrying strains was 14.9 and 6.1% in NICU I and II, respectively. These data were in accordance with our previous results, which were carried out on numerous *E. coli* strains isolated from different sources: antigen K1 was found in 11.0%, K5 in 6.6% [36]. The values were 56% and 3% in CSF. Antigens K1 and K5 occur together frequently with aerobactin production [38]. In this study K1 and Aer+ markers occurred together only in 3.3%, K5 and Aer+ markers in 4.7%. Because of the frequent occurrence of potentially pathogenic strains, the complex typing examinations are reasonable by drawing attention to the turning up of sepsis or meningitis. Colonization can be studied better by using phage type or pattern and plasmid profile examinations, because these methods give a help in further subdivision of serotypes. Complex examinations on *E. coli* strains showed the increase of serotypes O6:H–, O6:H1, O19:H– in NICU I and O4:H–, O6:H1 in NICU II.

Plasmid profile of *E. coli* strains was determined in 28% of the strains, therefore conclusions cannot be drawn, whether plasmid profiles of certain serotypes are homogeneous. *Klebsiella* infection was more frequent in NICU I than in NICU II. There was a difference in phage type distribution also in the two units, other phage types were present in NICU I (XI.A2), than in NICU II (II.A1). Phage type XI.A2, occurring in NICU I, and phage type II.A1, in NICU II could be isolated from nose, throat, ear, navel samples and environmental specimens. Forty percent of *Klebsiella*

strains were examined for plasmid profile. Homogeneous (I.A22) and heterogeneous (I.B1) plasmid profiles were found within one phage type.

Morgan et al. [39] examining 5 *Klebsiella*, 2 *E. coli* and 2 *E. cloacae* strains determined 112, 85, 22, 7, 3 plasmid profiles, they found only 112 Md carrying strains in transfer experiments. Among our isolates, a 112 Md plasmid-carrying strain was found in 85% and 44.4% in NICU I and II, respectively and this size was in accordance with the observations on *Klebsiella* and *E. coli* of Morgan et al. [39]. The presence of a 140 Md plasmid was also frequent, it being carried in 21.5 and 7.4% in NICU I and II, respectively, the plasmid of weight 60–70 Md occurred in 21.5 and 55.5% in unit I and II, respectively. The *Klebsiella* strains found in the two units differed both in phage types and plasmid profiles.

Plasmid of size 112 Md was not found in *E. coli*, 140 Md plasmid was demonstrated in O4:H1, O40:H55 strains. *P. aeruginosa* strains occurred less frequently than *E. coli* and *Klebsiella*, *P. aeruginosa* was carried by the infants in 12.6 and 20.4% in NICU I and II, respectively, it was isolated from nose, throat, ear, eye, navel samples in NICU I, and from nose, throat, wound, sputum and blood samples in NICU II. The strains were heterogeneous in respect of sero-, phage- and pyocin type and plasmid profile, for characterization serotyping supplemented with pyocin typing proved to be reliable. Determination of plasmid profile was not so effective, because the strains were plasmid free in 50%. This result is in accordance with the published data [40]. *S. aureus* was isolated infrequently (7.0 and 7.2% of the infants in NICU I and II, respectively). The phage patterns of the *S. aureus* strains were different, only the strains derived from 4 infants belonged to the same phage pattern (84 group III).

Table XV

Association of E. coli, Klebsiella, P. aeruginosa and S. aureus strains in the same newborn

Unit of examination	NICU I	NICU II
<i>E. coli</i> + <i>Klebsiella</i>	16	11
<i>E. coli</i> + <i>P. aeruginosa</i>	2	5
<i>E. coli</i> + <i>S. aureus</i>	2	2
<i>Klebsiella</i> + <i>P. aeruginosa</i>		2
<i>Klebsiella</i> + <i>S. aureus</i>	2	1
<i>E. coli</i> + <i>P. aeruginosa</i> + <i>S. aureus</i>		1
<i>Klebsiella</i> + <i>P. aeruginosa</i> + <i>S. aureus</i>		1
Mixed pattern	No. 22	23
	% 6.1	11.2

Table XIII summarizes the occurrence of *E. coli*, *Klebsiella*, *P. aeruginosa* and *S. aureus* strains according to the number and percentage of the infants. In NICU I the *Klebsiella* was more frequent whereas in NICU II *E. coli* and *P. aeruginosa* was more

common than in NICU I. The incidence of *S. aureus* was low in both units. Two or more of the above bacteria were found in 22 (6.1%) infants in NICU I, and in 23 (11.2%) infants in NICU II (Table XV).

In NICU I 267, in NICU II 174 infants were treated with antibiotics. Table XVI shows the administered antibiotics according to the number of infants. Because the antibiotics were also employed in combination, the number of the used antibiotics were not identical with the number of infants.

Examining the connection between the frequency of the used antibiotics and the resistance of the isolated *E. coli*, *Klebsiella*, *P. aeruginosa* and *S. aureus* strains, the data of Table XVI and Figs 3–6 were compared. Table XVI shows that penicillin derivatives were used in NICU I in 2.5–65.2%, in NICU II in 0.5–77.2%. There is no significant difference in the therapeutical use of penicillin derivatives in the two units. Resistance to penicillin derivatives of *E. coli* and *Klebsiella* strains was nearly the same in the two units. The rate of resistance was higher among *P. aeruginosa* strains in NICU I and *S. aureus* strains in NICU II.

Table XVI

Antibiotic treatment of infants

Antibiotic	NICU I		NICU II	
	No.	%	No.	%
Penicillin	40	14.5	3	1.7
Oxacillin	180	65.2	—	—
Azlocillin	37	13.4	5	2.7
Mezlocillin	18	6.5	1	0.5
Ampicillin	41	14.9	139	77.2
Methicillin	7	2.5	12	6.7
Cephalexin	5	1.8	—	—
Cefuroxime	16	5.8	3	1.7
Cefoperazone	2	0.7	—	—
Ceftriaxone	1	0.4	2	1.1
Cefamandole	—	—	3	1.7
Cefoxitin	10	3.6	—	—
Cefotaxime	3	1.1	46	25.5
Gentamicin	233	84.4	4	2.2
Amikacin	40	14.5	—	—
Tobramycin	—	—	81	45.0
Netilmicin	—	—	21	11.7
Nitrofurantoin	1	0.4	1	0.5
Lincomycin	7	2.5	—	—
Total No. of newborns	267		174	

Cephalosporin derivatives were used in 0.4–5.8% in NICU I and in 1.1–25.5% in NICU II. Resistance to first and second generation cephalosporins among *E. coli* strains was a bit higher in NICU I, except MA, and also among *Klebsiella* strains. There was not significant difference in the rate of resistant *P. aeruginosa* strains in the two units. The resistant *S. aureus* strains were more frequent in NICU II. There was not essential difference in the use of cephalosporins and resistance in the two units. Although cefotaxime therapy was employed in 1.1% in NICU I, and in 25.5% in NICU II, resistance to cefotaxime was not found among *E. coli* strains in either unit. Cefotaxime resistant *Klebsiellae* occurred in NICU II in 1.5%. *P. aeruginosa* strains were cefotaxime sensitive in NICU I, and resistant in 3.6% in NICU II. Resistance to cefotaxime was not found among *S. aureus* strains in either units. Aminoglycoside derivatives were administered in 14.5–84.5% in NICU I and in 2.2–45.0% in NICU II. Resistance developed in accordance with the administration: resistance to GM in *E. coli* was more frequent in NICU I, in case of *Klebsiella* strains resistance to all aminoglycosides was more frequent in NICU I, resistance of *P. aeruginosa* and *S. aureus* was nearly the same in both units,

Fryklund et al. [41] examining 953 infants in neonatal units, found that *E. coli*, *Klebsiella* and *Enterobacter* spp. occurred in stool in 23.0%. Connection was not found between antibiotic therapy and colonization of nosocomial enteric strains. Tullus and Burman [42] published that the colonization of antibiotic resistant strains was higher among the untreated infants and they stated that infants became rather the source of nosocomial strains, than recipients as a consequence of antibiotic therapy.

Table XVII

Connection between antibiotic treatment and incidence of examined bacteria

Antibiotic treatment	Bacteriologically	NICU I		NICU II		Total	
		No.	%	No.	%	No.	%
Treated	Positive	80*	30.0	59**	34.0	139	31.5
	Negative	187*	70.0	115**	66.0	302	68.5
Total		267	100.0	174	100.0	441	100.0
Not treated	Positive	119*	53.8	110**	66.3	229	59.2
	Negative	102*	46.2	56**	33.7	158	40.8
Total		221	100.0	166	100.0	387	100.0

*, ** $p < 0.001$

The spread of resistance to gentamicin and tobramycin among enteric bacteria was 2.8% in mixed wards during a one-year period [43]. Molecular examinations on plasmid-determined gentamicin resistance among nosocomial *P. aeruginosa* and *Klebsiella* strains were described by Rubens et al. [44]. Colonization of multiple resistant *Klebsiella pneumoniae* was described by genotypic- and plasmid profile determination in neonatal ward [45].

In our examinations in the two NICUs the rate of occurrence of the examined bacteria isolated from the treated and untreated infants was compared. It was found, that in the group treated with antibiotics bacterial positivity was 30.0% in NICU I, and 34.0% in NICU II, compared to the total number of cases. The examined bacteria were isolated in a significantly lower rate in the group, treated with antibiotics than in the untreated group (54.0 and 66.0%, respectively). There was no significant difference between the bacterial positivity of infants treated with antibiotics in the two units ($p > 0.5$), in the untreated group positivity was more frequent in NICU II ($p < 0.01$) (Table XVII). This data are in accordance with results presented in Table XIII, that *E. coli* and *P. aeruginosa* were isolated from infants in a higher degree in NICU II, among the less frequent *Klebsiella* isolates the colonization of the same type was observed in 50.0%, the types of isolates were heterogeneous in NICU I.

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THE ROLE OF FREE RADICALS AND ANTIOXIDATIVE ENZYMES IN ERYTHROCYTES AND LIVER CELLS IN THE COURSE OF *PLASMODIUM BERGHEI* AND *PLASMODIUM VINCKEI* INFECTION OF MICE*

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Blood schizontocidal test of D₀ + D₃ type revealed different characteristics of the *Plasmodium berghei* and *Plasmodium vinckei* infection. Both types of the rodent plasmodia kill the untreated mice. Chloroquine treatment alone does not prevent the death of the *P. berghei* infected animals and they die at a low level of parasitaemia. The animals cured with chloroquine plus MAP survive. The infection with *P. vinckei* produces a high level of parasitaemia and the chloroquine treatment alone prevents the death of mice. The difference in the pathogenic characteristics between *P. berghei* and *P. vinckei* is manifested in the results measuring the kinetics of the activity of antioxidative enzymes in the red blood and liver cells of the infected mice: lipid peroxidation (LPO), superoxide dismutases (SOD), glutathione peroxidase (GP), catalase (CAT) and reduced glutathione (GSH). The rapid increase of the LPO in the RBC in particular in the *P. vinckei* infected animals indicates the prevailing role of the membrane detoxification process. A continuous increase in the activity of enzymes of cytoplasmic origin, e.g. SOD and GP was also observed. A powerful increase in GSH distinguishes the erythrocytes of *P. vinckei* infected animals. Similar but not identical data characterize the enzyme activities of the liver cells of the plasmodia infected animals.

In an previous paper [1] we reported the protective effect of a new synthesized polypeptide, MAP-1987 in combination with chloroquine in *P. berghei* infected mice. This compound prevents the haemolysis of the parasitized cells and cures the lethal infection given together with chloroquine. This effect does not apply to *P. vinckei* infection.

*This paper was written in honour of the memory of Professor Zoltán Alföldy (1904–1992) director emeritus of the Institute of Microbiology Semmelweis University Medical School in Budapest (Hungary)

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There are evidences about the role of free oxygen radicals, the reactive oxygen species (ROS) in the lysis of the parasitized erythrocytes [2–4] and its interpretation of immunity to malaria parasites [5]. The ROS play an important role in the damage of the different cell types and are associated with many injuries of the organism [6]. However, the cells develop protective mechanisms against the oxidative stress by detoxification and damage repair reactions.

Concerning the membrane vulnerability and antioxidants of the erythrocytes (RBC), experimental data and theoretical considerations [7] argue for the potential hazards of aerobic environment. The RBC are laden with iron and unsaturated lipids of its membrane are continuously subjected to oxygen. The evolution provided the RBC with antioxidant defense and reductive mechanisms which are incomplete due to the limited capacity against the oxygen challenge. The oxygen challenge begins with the generation of superoxide (O_2^-) and its dismutation yields peroxide (H_2O_2). In the next step the reducing agents generate hydroxyl radical ($HO^\cdot$) which is highly reactive with many organic molecules.

The antioxidant mechanisms of the RBC are the detoxification and damage repair reactions. The detoxification reactions take place at the cytoplasmic and membrane level. The cytoplasmic antioxidants are the superoxide dismutases (SOD) producing H_2O_2 , which is eliminated by catalase or glutathione (GSH) and GSH peroxidase. The only detoxification mechanism located in the RBC membrane is the lipid peroxidation (LPO) which is an autocatalytic reaction and convert the lipid peroxy radicals ($LOO^\cdot$) to lipid hydroperoxides (LOOH) and other decomposition and end products.

The oxidative damage repair mechanism among others are the metHb ($HbFe^{+++}$) reduction and the glutathione reductase mechanism which restore the reduced glutathione (GSSG) resulting from the above-mentioned detoxification reaction. The GSSH peroxidase reduces some lipid hydroperoxides too at the expense of GSH. The lipid hydroperoxides, products of the membrane lipid peroxidation initiated by free radicals (e.g. $HO^\cdot$), terminate by vitamin E as a membrane protector.

Other defense mechanisms might play role in malaria immunity. There are the heme-containing enzymes known as the cytochromes P450. They protect living organisms against the toxic chemicals adding oxygen to a wide range of compounds [8, 9].

We are reporting in this paper about the investigation on the antioxidant and detoxicating enzymes in the RBC and liver microsomes of *P. berghei* and *P. vinckei* infected mice related to the course of parasitaemia and with special concern to the protective and/or synergistic action of the MAP-1987 with chloroquine.

Materials and methods

Chemicals and reagents. The MAP-1987 was supplied by BioPharm, Budapest. Its characteristics are described in the previous paper [1]. The chloroquine phosphate was supplied by the WHO, MAP/RTI. The reagents used for the measurements were products of Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Sigma (St. Louis, USA), Calbiochem (Lucern, Switzerland), VEB Laborchemie (Jena, Germany) and Reanal (Budapest, Hungary) of the purest quality.

Parasites and host. *P. berghei* N strain and the *P. vinckei* were maintained in CFLP Swiss mice by weekly serial passage using a standard inoculum which produces uniform disease fatal to 100% of

untreated animals with a survival time of 6 days for *P. berghei* and 5 days for *P. vinckei*. Test animals weighed 22–25 g. All animals in any given test were approximately of the same age.

Blood schizontocidal test. The $D_0 + D_3$ type of the test [10] was performed in the experiments. Test animals received an intraperitoneal (i.p.) injection of 0.5 ml heparinized heart's blood with highly parasitized RBC and diluted to contain the optimal infecting dose killing all mice in the control group on the same day within few hours. The standardization of the infecting dose is described in the previous paper [1]. The compounds were also i.p. administered. The parasitaemia was determined daily in the first week, then every two days during the experiment. The survival time of the mice was registered.

Preparation of microsomes. Microsomes were prepared from 0.5 g liver as previously described [11]. The homogenate in 5 ml 25 mM sucrose and 5 mM Tris-Cl pH 7.5 was centrifuged at 600 for 10 min, then at 9000 g for 10 min. The supernatant was again pelleted at 17 000 g for 10 min and the pellet was discarded.

The microsomes were precipitated with 25 ml 0.5 mM CaCl_2 and 0.3 mM MgCl_2 solution and kept in ice-cold water for 15 min followed by centrifugation at 2000 g. The pellet was suspended in 6 ml 25 mM KCl in 8 mM Tris HCl, pH 7.5 with 1.5 ml glycerol and 0.1 ml TRITON X-100. The microsome suspension was aerated with CO . The whole procedure ran under cooling. The concentration of microsomal and of RBC lysates proteins were determined by the method of Lowry et al. [12].

Preparation of erythrocyte lysate. The RBC of heparinized heart blood were washed with PBS three times and haemolyzed with 10 volume distilled water.

The aliquots of lysate were frozen at -20°C for the enzyme determinations. For measuring the total SOD activity, one aliquot was pretreated with one volume ethanol and chloroforme mixture (2:1) and after separation the haemoglobin-free supernatant was used.

Enzyme determinations. *Superoxide dismutase* (SOD) (EC 1.15.1.1.) was measured by the method of Misra and Fridovich [13] and the Mn-SOD according to Beauchamp and Fridovich [14]. In principle, the inhibition of epinephrine adrenochrome transformation was determined. One unit enzyme inhibits the transformation by 50% in 1 min. The Mn-SOD was measured when reaction mixture contained 60 mM KCN. The difference between the measured activities of total- and Mn-SOD gives the quantity of the Cu, Zn-SOD.

Catalase (EC 1.15.1.6) activity was measured according to Beers et al. [15] at 240 nm. One Bergmeyer activity unit means the decomposition of 1000 mg H_2O_2 as substrate at 25°C in 1 min.

Reduced glutathione (GSH) was determined according to Sedlak and Lindsay [16]. In the reaction mixture the non protein-bound SH groups give a yellow coloured reaction with the 5,5-dithiobis(2-nitrobenzoic acid) measurable at 412 nm.

Glutathione peroxidase (E.C.1.11.1.9) activity was measured according to Chiu et al. [17] with Ellman reagent. The activity was calculated from the differences between the content of GSH before and after the enzyme reaction.

Lipid peroxidation was determined by the method of Placer et al. [18]. In this reaction the malondialdehyde (MDA) as an endproduct of lipid peroxidation gives a reddish yellow coloured reaction with 2-thiobarbituric acid and it is measured at 532 nm. The values are related to nM MDA/ml RBC haemolysate or nM/g tissue homogenate.

Cytochrome P-450 was prepared from microsomes of liver cells and measured by the method of Greim [19]. The method is based on the carbon monoxide sensitivity of the haemoproteins. The CO-haemoproteins were reduced with $\text{Na}_2\text{S}_2\text{O}_4$ and the absorbency measured between 400 and 500 nm. The spectrum has in general two peaks; the first one is identical with the cytochrome b5 and the second one is the cP-450.

The enzyme activities were measured by sacrificing daily 3–5 animals of a group of mice infected either with *P. berghei* or *P. vinckei*. The specific activity was calculated, based on the protein content of the samples. The changes in the enzyme activity during the course of the infection are related to the normal values of the non-infected animals represented in the figures as base-line. The increases and decreases of the enzyme activity are calculated in percent of normal values.

Results

Stocker et al. [20] examined the protection of mouse RBC parasitized with *P. vinckei* against activated oxygen species in relation to the intraerythrocytic parasite load. The parasitized RBC were separated in density gradient into 6 fractions of increasing parasite content. Suthipark et al. [21] investigated the SOD activity of the isolated and purified *P. berghei* at the time of high parasitaemia. Contrary, our intention was to follow up the dynamic of the antioxidant enzyme during the whole period of the infection of *P. berghei* and *P. vinckei* in the control group of mice as well as of groups treated with MAP-1987, chloroquine and chloroquine + MAP. The rate of parasitaemia was also measured and these results are summarized in Figs 1 and 2.

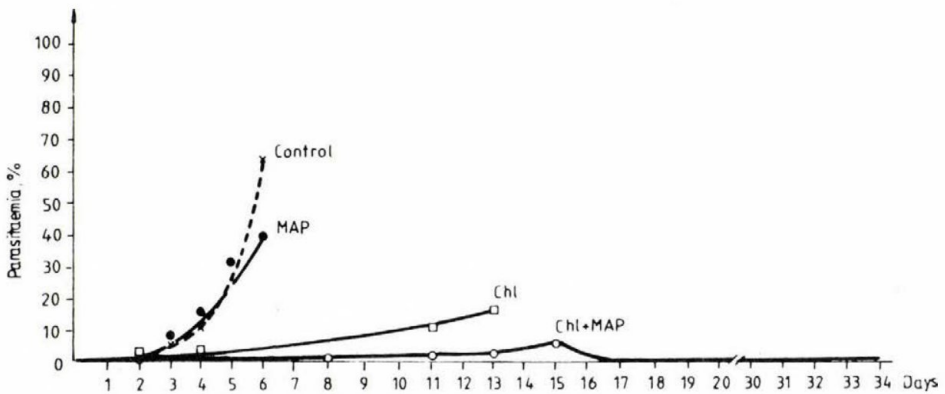


Fig. 1. The course of parasitaemia in *P. berghei* infected mice in the D₀ + D₃ experiment

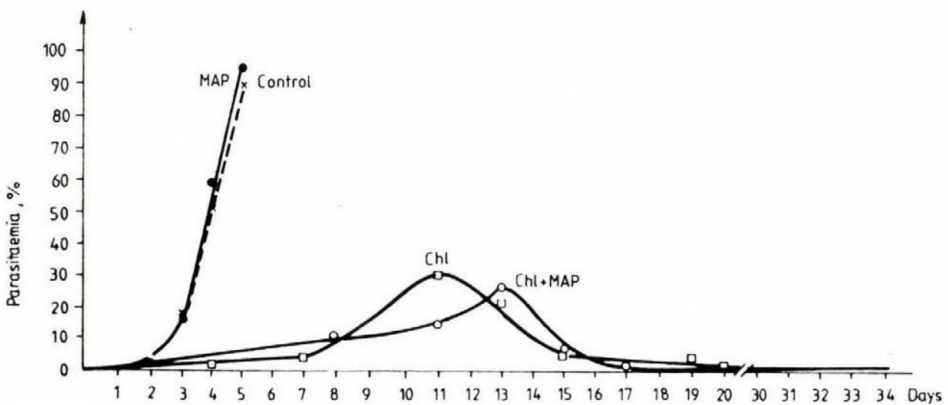


Fig. 2. The course of parasitaemia in *P. vinckei* infected mice in the D₀ + D₃ experiment

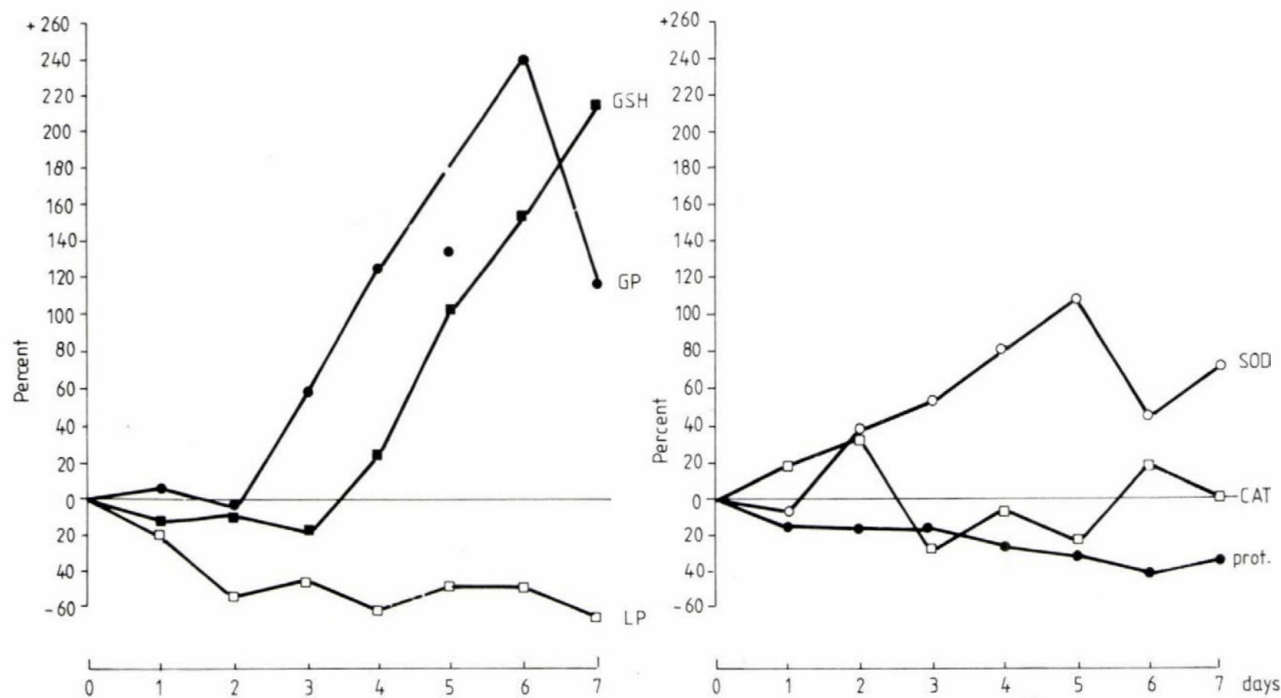


Fig. 3. The activity of antioxidative enzymes in the erythrocytes parasitized with *P. berghei*

Figure 1 shows the course of parasitaemia in *P. berghei* infected mice. In the control group the parasitaemia increased up to 70% before the day of the death of animals. MAP treatment decreased the parasitaemia, inhibited the lysis of the RBC [1] and the animals died some hours earlier than those of the control group. The chloroquine treated mice survived until the 14th day with slowly increasing parasitaemia and MAP + chloroquine cured all of the infected animals; the curve of parasitaemia had a only low peak at the 15th day [1].

Figure 2 shows the course of the parasitaemia in *P. vinckei* infection. The parasitaemia increased up to 90% of that shown in the control group as well as in the MAP-treated animals on the 4th day, and MAP did not prevent the lysis of RBC. Chloroquine and chloroquine + MAP treatment equally cured the infection with a lower peak of parasitaemia on the 11th and 13th days. The data about the course of parasitaemia of the infected animals offered an opportunity for comparative study of dynamic activity of the antioxidant enzymes in the unfractionated RBC.

Figure 3 summarizes the changes in the enzyme activity during the course of *P. berghei* infection. Concerning the cytoplasmic enzymes of detoxification processes in the RBC we observed a continuous increase of the SOD activity up to 100% until the 5th day of infection. This is accompanied by a more powerful increase of the oxygen radical eliminating GSH and H_2O_2 decomposing GSH peroxidase. The curve of catalase activity is moving around the base line indicating that in the H_2O_2 elimination process the GSH system is the prevailing factor. The only detoxification mechanism specifically located in the RBC membrane is the lipid peroxidation. The activity of LPO in the *P. berghei* infected RBC is remarkable decreasing. In contrast, this is not the case in the *P. vinckei* infected RBC shown in Fig. 4.

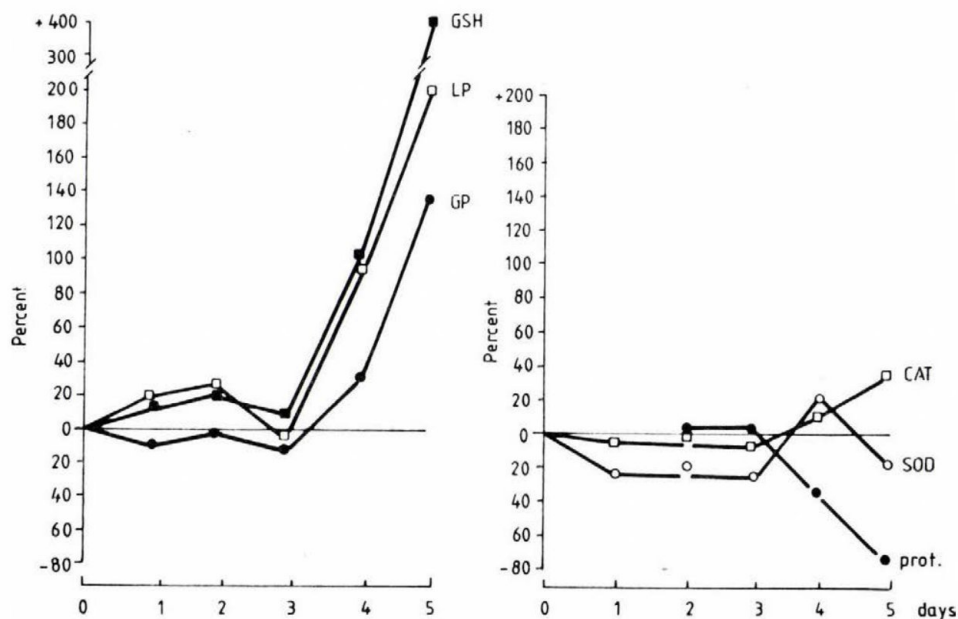


Fig. 4. The activity of the antioxidative enzymes in the erythrocytes parasitized with *P. vinckei*

The activity of the SOD is low and it decreased until the 3rd day. At this time began to increase moderately the SOD and rapidly the GSH as well as the GSH peroxidase activity. The most striking was the rapid increase of the LPO indicating the distinguished role of the membrane detoxification process in the *P. vinckei* infected RBC. The liver being involved in the life cycle of plasmodia we investigated the course of antioxidant enzyme activities in liver homogenates and microsomes. The results of experiments performed on mice infected with *P. berghei* and *P. vinckei* are summarized in Figs 5 and 6.

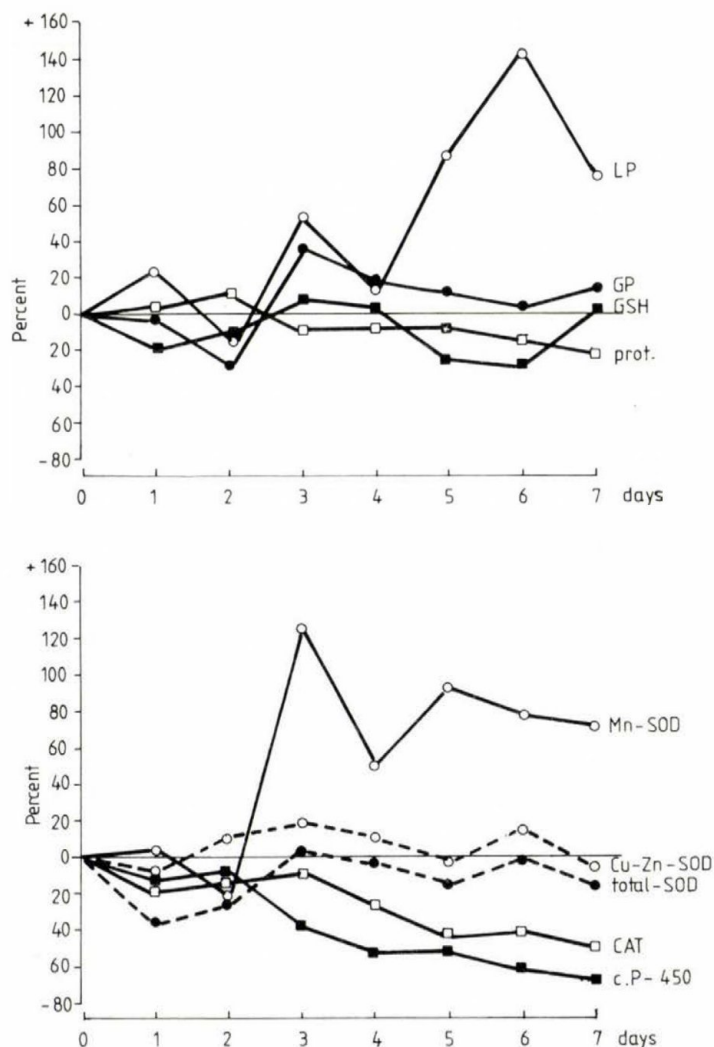


Fig. 5. Activity of antioxidative enzymes in the liver cells of mice infected with *P. berghei*

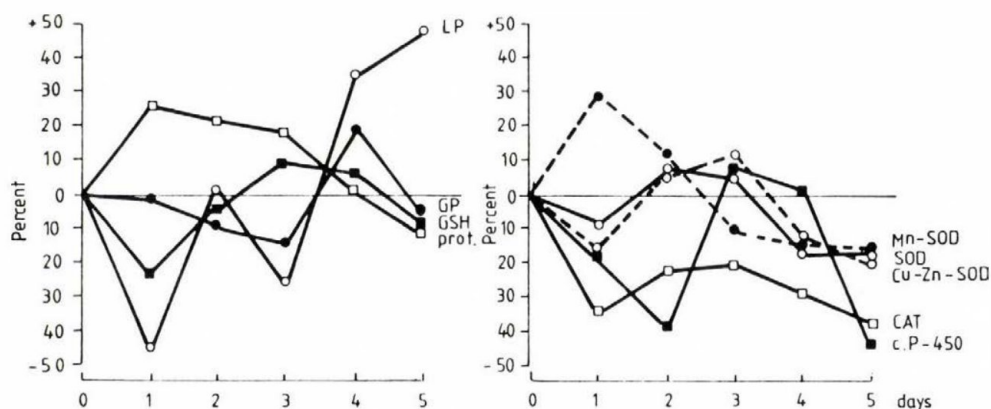


Fig. 6. Activity of antioxidative enzymes in the liver cells of mice infected with *P. vinckei*

Figure 5 shows that only the Mn-SOD activity is increasing under the cytoplasmic detoxifying enzymes and the others are moving around the base line (normal values). The catalase and the c.P-450 activity decrease significantly. The membrane located LPO shows a high activity increasing by 150% on the 6th day with a cyclic character. Figure 6 concerns the antioxidant enzyme activity of the liver of *P. vinckei* infected mice. The cyclic character of the curves is appreciable and its periodicity changes by 2 days. Only the membrane located LPO is increasing and the c.P-450 level decreases significantly.

Discussion

The results of the first experiments [2-4] suggested that ROS causes the intraerythrocytic death of parasites (*P. vinckei*). Other studies have shown [22, 23] that GSH stability in the parasitized RBC was enhanced and questioned the role of oxidative stress in malaria. These type of experiment were performed relating the GSH levels and enzyme activities in term of protein [22] or haemoglobin concentration [23]. Others expressed the results of this type of experiment on a per cell basis, e.g., the SOD activity in the mouse RBC infected with *P. berghei* [23] and the antioxidant enzyme in the mouse RBC infected with *P. vinckei* [22]. They found that the *P. berghei* infected RBC have increased SOD activity. Experiments done with *P. vinckei* RBC [20] resulted that increase in parasite load was accompanied by a decrease in the activities of the enzyme SOD, catalase, GSH peroxidase, glutathione reductase and NADH-methaemoglobin reductase in the RBC lysate. In contrast, the total amount of GSH increased in the highly parasitized RBC fractions as well as the LPO. The results indicated that the oxidative stress is observable on all RBC during the

P. vinckei infection. The membrane of the host cells seems to be better protected than those of the parasites.

It is well known that the plasmodia and the host cells have independent metabolic pathway [24]. In spite of this, *P. berghei* in mice was found to derive a substantial amount of SOD activity from the host cell cytoplasm [25].

In the damage repair mechanism, the reduction methHb (HbFe⁺⁺⁺) is a critical element, even the iron salt and heme compounds take part in the decomposition of the LOOH [26, 27]. In addition, the membrane iron and O₂ potentially allows the reinitiation of lipid peroxidation bypassing efficacy of vitamin E [7].

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TYPING OF COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM IMMUNOCOMPROMISED PATIENTS*

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A total of 3121 coagulase-negative staphylococcal strains sourced from clinical samples were characterized during a 4-year period. Biotype, antibiotic resistance pattern, phage pattern and slime production was determined. Plasmid profile analysis was performed on related isolates. Thirty percent of strains originated from the Bone Marrow Transplantation Unit, The National Institute of Haematology, Blood Transfusion and Immunology (NIHBTI), Budapest. *Staphylococcus epidermidis* occurred most frequently (48.8% in total, 58.2% sourced from NIHBTI). Total bacteriophage typability was 75.9%, and 603 phage patterns were observed. NIHBTI isolates differed in the incidence of multiply resistance, slime positivity and average frequency of phage patterns from the total suggesting spread of a selected hospital population. Statistical analysis of data obtained by typing showed no predominance of any endemic clone: the strains colonizing the immunocompromised patients and isolated from staff and inanimate environment differed from each other in biotype, phage pattern, antibiotic susceptibility, slime production and/or plasmid profile.

Coagulase-negative *Staphylococcus* (CNS) species are considered to be normal inhabitant of the skin and mucous membranes [1]. Under certain circumstances, CNSi as opportunist pathogens may cause infections in patients with compromised immunity including neonates and associated with implanted medical devices [2–6].

The ubiquitous nature of CNS has posed problems in deciding the significance of an individual isolate as a causative agent of infection. Assessment is based on the clinical condition of the patient and the presence of closely related isolates in subsequent specimens taken from the patient [7, 8]. The isolation of the same CNS strain on several occasions from the same patient supports clinical relevance of the isolates or its isolation from different patients suggests the presence of a hospital infection. Outbreaks of nosocomial infections caused by epidemic CNS strains have been reported [8–13]. Epidemiological studies are based on determination of the

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biotype, antibiotic susceptibility pattern, phage type, detection of extracellular products such as slime factor [14–16]. Molecular techniques such as exoprotein [5] or plasmid profile [14, 16–18], DNA restriction endonuclease fingerprinting [19, 20], pulsed field electrophoresis [21], ribotyping [22] or lectin-binding [23] and serological assays [8, 24, 25] have recently been applied in recognition of hospital-acquired infections of CNS.

Typing of CNS isolates was introduced in 1989 at the Bacteriophage Department of B. Johan National Institute of Hygiene, the national reference laboratory for bacterial typing, Budapest, Hungary. To characterize of isolates, phage typing, biotyping, antibiotic sensitivity testing, detection of slime factor and plasmid-profile analysis were applied. Experiences obtained during this four-year period and analysis of strains isolated from a bone-marrow transplantation care unit are summarized in this report.

Materials and methods

Bacterial strains. A total of 3121 CNS strains were typed. Strains were isolated by 5 county institutes of the Public Health Service and 20 hospital laboratories. Bacterial colonization of immunocompromised patients hospitalized at the Bone Marrow Transplantation Unit of the National Institute of Haematology, Blood Transfusion and Immunology (NIHBTI), Budapest was checked two times weekly by swab specimens. The following samples were cultured routinely: skin, nose, and vagina/foreskin, respectively. The species identification followed the method of Kloos and Schleifer [26].

Table I

Determination of biotypes (BT) of coagulase-negative staphylococci

Numeral code	Acid from*			
	(X=) Lactose (Y=) Mannitol	Maltose Sucrose	Mannose Trehalose	
1	+	+	+	
2	+	+	–	
3	+	–	+	
4	–	+	+	
5	+	–	–	
6	–	+	–	
7	–	–	+	
8	–	–	–	

* Results of carbohydrate breakdown are grouped by Farmer's code [30]. Biotype (BT) is designated with two-figure numbers. First cipher (X) and second cipher (Y) of BT issue from grouping of reactions of lactose/maltose/mannose and mannitol/sucrose/trehalose, respectively [31].

Phage typing was carried out by Pulverer and colleagues' phage set [27]. Phages were used in routine test dilution (RTD) and also in hundred-fold concentration (100 RTD). Nontypable strains were retyped with phages of 100 RTD after a two-minute heat-treatment at 56 °C [28]. Two strains were regarded as different from each other if their phage patterns were distinct in at least one strong phage reaction (i.e. confluent, semi-confluent or +++ lysis) [29].

Biotyping was performed by results of six biochemical tests grouped according to Farmer [30, 31] (Table I).

Slime production was tested in Tryptone Soya Broth (Oxoid, London) [32, 33].

Antibiotic resistance pattern was determined by disk diffusion test [34] on Mueller-Hinton medium (Oxoid, London). The following set of Resistest antibiotic disks (Human, Gödöllő, Hungary) was used: penicillin G (P, 3 IU), oxacillin (OX, 10 µg), cefalexin (CX, 30 µg), cefamandole (MA, 30 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg) tobramycin (TM, 30 µg), erythromycin (E, 10 µg), lincomycin (L, 10 µg), co-trimoxazole (SXT, 25 µg), and vancomycin (VA, 50 µg).

Plasmid profile analysis. Plasmid DNA was prepared according to Coleman et al. [35]. DNA electrophoresis was performed in 0.8% agarose (Sigma, St. Louis) in tris/borate buffer [36].

Characterization of the strains. For statistical evaluation of results of typing, grouping of phage patterns and antibiotic resistance phenotypes, the method described previously [37], was used.

Table II

Clinical origin of coagulase-negative staphylococcal isolates between 1989 and 1992

Sample	Number (per cent) of isolates					
	All		NIHBTI ¹		Other	
Nose	199	(6.4)	129	(13.5)	70	(3.2)
Throat	96	(3.0)	40	(4.2)	56	(2.6)
Skin	220	(7.0)	205	(21.5)	15	(0.7)
Wound	211	(6.8)	4	(0.4)	207	(9.6)
Intraabdominal	97	(3.1)	0		97	(4.5)
Blood	729	(23.4)	44	(4.6)	685	(31.6)
Catheter	276	(8.8)	171	(17.9)	105	(4.8)
Urine	517	(16.6)	52	(5.4)	465	(21.5)
Semen	316	(10.1)	0		316	(14.6)
Genitals ²	192	(6.2)	167	(17.5)	25	(1.2)
Other ³	99	(3.2)	14	(1.5)	85	(3.9)
Environmental						
Personnel ⁴	157	(5.0)	117	(12.3)	40	(1.8)
Air	12	(0.4)	12	(1.2)	0	
Total	3121	(100)	955	(100)	2166	(100)

¹ Bone Marrow Transplantation Unit of National Institute of Haematology, Blood Transfusion and Immunology, Budapest, Hungary

² vagina and foreskin

³ eye, ear, heart valve, cerebrospinal fluid, sternal punctate, synovial fluid, bile, faeces, milk

⁴ nose and hand

Table III

Percentage distribution of coagulase-negative staphylococcal isolates typed between 1989 and 1992 (%)

Species	Source		
	NIHBTI*	Other	All
<i>S. epidermidis</i>	58.2	44.7	48.8
<i>S. haemolyticus</i>	22.7	22.9	22.9
<i>S. homini</i>	5.4	9.8	8.5
<i>S. warneri</i>	1.2	4.3	3.4
<i>S. saprophyticus</i>	0.2	3.6	2.5
<i>S. simulans</i>	0.9	2.2	1.8
<i>S. capitis</i>	1.0	0.3	0.5
<i>S. xylosus</i>	0.1	0.3	0.3
<i>S. cohnii</i>	0.1	0.3	0.2
<i>S. lentus</i>	—	0.04	0.03
Unidentified	9.9	11.3	10.9
Total	100	100	100

* Bone Marrow Transplantation Unit of National Institute of Haematology, Blood Transfusion and Immunology, Budapest, Hungary

Results

Table II shows the origin of the isolates. A total of 955 strains (30%) sourced from the Bone Marrow Transplantation Unit of NIHBTI. Species distribution of the isolates showed a predominance of *S. epidermidis*. This species occurred 15% more frequently in NIHBTI than in other sources (Table III).

Occurrence of slime producer and also of multiply resistant strains was considerably higher among isolates from NIHBTI and the correlation of slime positivity and multiple drug resistance is more distinct in this group (Table IV). Average of resistance determinants harboured was 3.3 in total, 4.1 in NIHBTI strains and 2.9 in the rest of the collection, respectively.

Phage typability of isolates is documented in Table V. There was no difference between the total typability of strains of NIHBTI and of other origin; frequency of strong phage reactions in RTD was lower than that of the total. Comparison of number and average of phage patterns (Table VI) demonstrated NIHBTI isolates to be more homogeneous than randomly collected isolates were.

Table IV

Percentage frequency of slime producer (S+) and multiply resistant (RR) coagulase-negative staphylococcal isolates between 1989 and 1992

	<i>S. epidermidis</i>	Other species	Summarized
All			
Frequency	48.8	51.2	100
S+	58.5	22.7	40.2
RR	62.9	54.6	58.6
S+ and RR	44.3	13.9	28.7
NIHBTI*			
Frequency	58.2	41.8	100
S+	70.8	20.6	49.8
RR	76.9	77.9	77.4
S+ and RR	59.5	17.5	41.9
Other source			
Frequency	44.7	55.3	100
S+	51.6	23.5	35.9
RR	54.9	47.0	50.4
S+ and RR	35.6	12.6	22.9

* Bone Marrow Transplantation Unit of National Institute of Haematology, Blood Transfusion and Immunology, Budapest, Hungary

Table V

Bacteriophage typability of coagulase-negative staphylococcal isolates between 1989 and 1992

Species	Origin	Typability (%)			
		RTD	100 RTD	100 RTD/Heat treatment	Nontypable
All	All	41.1	30.8	3.9	24.1
	NIHBTI*	28.9	44.2	5.3	21.5
	Other	45.2	26.4	3.4	25.0
<i>S. epidermidis</i>	All	26.3	52.7	7.1	13.9
	NIHBTI*	27.2	55.5	5.8	11.5
	Other	25.3	49.9	8.3	16.5

* Bone Marrow Transplantation Unit of National Institute of Haematology, Blood Transfusion and Immunology, Budapest, Hungary

Table VI

Number and average incidence of phage patterns among coagulase-negative staphylococcal isolates between 1989 and 1992

	All species			<i>S. epidermidis</i>		
	All sources	NIHBTI*	Other source	All sources	NIHBTI*	Other source
Number of strains	3121	955	2166	1525	556	969
Number of phage patterns	603	120	578	217	48	146
Average of phage patterns**	5.2	7.9	3.7	7.0	11.6	6.6

* Bone Marrow Transplantation Unit of National Institute of Haematology, Blood Transfusion and Immunology, Budapest, Hungary

** Average incidence was calculated as number of strains/number of phage patterns

During the first period (1987–1989) 152 isolates of 14 patients at the NIHBTI were typed. *S. epidermidis* (69 isolates, 45%) were collected from samples of 11 patients. Single lysis with phage Ph13 occurred in 75% colonized 7 patients and a long pattern with phages Ph10/ Ph13/ Ph15/ U4/ U15/ U16/ U20/ U33/ U46 in 19% of *S. epidermidis* isolates of 4 patients. *S. haemolyticus* (68 isolates, 45%) was in 66% nontypable distributed in 3 patients. Although isolates from individual patients differed in majority from other patients' isolates in at least one character, 18 slime producer, P, TE, TM and SXT resistant strains of *S. epidermidis* biotype 16 were lysed by only phage Ph13; these strains were cultured from samples of 5 patients. By plasmid pattern, each patient's strains differed from those colonizing other individuals. In this period, environmental strains were not obtained.

In the second period (1990), 54 CNS isolates of 3 patients and the hospital environment were examined. *S. epidermidis* predominated (72%), 39 isolates of two patients and of inanimate environment belonged into this species. In phage typing, 69% of them was nontypable (isolates from two patients and the environment). *S. epidermidis* BT26, nontypable with resistance pattern P TM SXT, slime positive occurred in specimens of one patient and in 6 environmental samples.

Analysis in the third period (1992) comprised 136 CNS isolates of 4 patients, the staff and the hospital environment. *S. epidermidis* was most frequently isolated: 87 isolates (64%) from all four patients, from staff members and environmental samples. Sixty-six percent of *S. epidermidis* isolates predominating in every source belonged to phage pattern Ph12/ Ph13/ Ph15/ U14/ U15/ U20. The strains with this phage pattern differed from each other in biotype, antibiotic resistance pattern, slime positivity and/or plasmid profile.

During the four years, CNSi isolated from samples of NIHBTI belonging not only to the examined accumulations were compared statistically. Only *S. epidermidis*

BT16 strains with phage pattern Ph5/ Ph9/ Ph10/ Ph12/ Ph13/ Ph15/ U4/ U14/ U16/ U20/ U33/ U46 were isolated from different samples during these period. Origin and characterization of 14 representatives are documented in Table VII. Antibiotic resistance patterns and plasmid profiles of these strains displayed considerable differences.

Table VII

*Characters of S. epidermidis biotype 16 strains with phage pattern
Ph5/ Ph9/ Ph10/ Ph12/ Ph13/ Ph15/ U4/ U14/ U16/ U20/ U33/ U46*

Strain	Isolation year	Patient/sample	Antibiogram				Plasmid profile (Md)
41/89	1989	H.R.	P	TE	TM	SXT	5.0 3.1 1.7
56/89	1989	V.Cs.	P	C	TE	TM SXT E L	15 5.8 5.3 4.9 3.1 1.5
159/89	1989	T.A.	P	TE			21 3.0
65/89	1989	K.L.	P	TE			
1043/90	1990	mattress	P	TE	SXT		3.1
1028/90	1990	air (boxII)	P	TE			3.0
1050/90	1990	gloves	P OX C	TE	TMSXT		18 3.0 2.6
922/91	1991	M.I.	P	C	TE	SXT	3.1 2.9
923/91	1991	M.I.	P	TE	SXT		3.1
124/92	1992	M.P.	P OX		TMSXT	E L	18 5.4 3.8 2.0
284/92	1992	T.Gy.	P	TE			13.2 3.1
299/92	1992	air (boxII)					
383/92	1992	air (boxI)	P OX C	TE	TMSXT	E L	23 3.1 1.8
388/92	1992	air (hall)	P	C	TE	SXT	3.0 2.8

Abbreviations: P – penicillin G, OX – oxacillin, C – chloramphenicol, TE – tetracycline, TM – tobramycin, SXT – co-trimoxazole, E – erythromycin, L – lincomycin

Discussion

Coagulase-negative staphylococci have been increasingly recognized as nosocomial pathogens in patients whose defences were compromised by an implanted foreign body or by immunosuppression [2, 4]. These bacteria present special hazards in patients treated in long-stay hospital wards, especially in intensive or transplantation care units. They are exposed to nosocomial infections due to microorganisms persisting permanently in the patients' flora and in the inanimate environment. The main problems of hospital epidemiology are the multispecies outbreaks, the patient-to-patient spread of these bacteria, and their increased resistance to antimicrobials. In particular, CNS colonization on immunocompromised patients was considered to be a contributing factor in higher mortality [3, 4, 11, 13, 14, 38].

A commensurate rise in interest in the study of the epidemiology of CNS-related infections has occurred and resulted in the application of various typing systems for investigations of CNS reservoirs and modes of transmission between patients and staff [9, 14, 16]. Antibigrams are done routinely by the clinical microbiology laboratories. The presence of a strain with a unique antibiogram could provide a marker for detecting similar strains. Because antibiograms are influenced by the acquirement or spontaneous loss of resistance determinants [14, 39], this method of typing is more likely to be useful only in short periods but not for retrospective analysis of epidemics. To determine resistance pattern, "class" antibiotics of each antibiotic group were chosen.

Biotyping, i.e. determination of biochemical profiles [14, 15] is based on intraspecific heterogeneity: positivity of several biochemical reactions ranges between 20–80%. Our method for subgrouping of CNS isolates is easy to perform and does not require special instrumentation [31].

For phage typing, Pulverer and co-workers' phage set [27] was used. Using this phage set, typability was 44% by Fridhandler et al. [40], 48% by Shimizu et al. [41], 58% by Brandis et al. [42], 69% by Staal et al. [29], 72% by Heczko et al. [43] and 80% by Nord and Sjöberg [44], without heat treatment. In our hand, total typability of the strains was 75.9%, and 603 phage patterns were observed.

Extracellular mucoid substance, so-called slime material, as a genetically stable marker, serving to adhere CNS cells to the implanted devices and for surviving in the presence of antibiotics [32] is applicable to characterize strains of epidemiological importance [14, 16]. In randomly selected collection, this marker was most frequent among strains of *S. epidermidis* (57%) [33].

Application of plasmid profile determination in epidemiological studies is limited as a labour and instrumentation-requiring method, and it may change due to newly acquired resistance determinants during an outbreak [14, 16]. Examining selected isolates, this method is frequently used for actual characterization of endemic isolates [5, 10, 13, 18] or to differentiate contaminants from clinically relevant strains [14, 17].

Summing up of the four-year-period typing demonstrates significant differences in characters of strains sourced from NIHBTI and those of other origin. The higher incidence of either slime producer or multiply resistant isolates, and the higher average of both resistance determinants and phage patterns in NIHBTI, especially within the predominating species *S. epidermidis*, suggested a spread of several hospital strains.

Species distribution differed considerably from those observed among hospital isolates [32]. Incidence of *S. hominis*, a normal flora composer [1], was low but that of *S. haemolyticus* was higher than in randomly selected collections. Only one *S. saprophyticus* strain was isolated. Slime positivity of *S. epidermidis* isolates was identical in both studies [32].

In our previous work, we gave account of the preliminary results on typing of CNS isolates sourced between 1987 and 1989 (period I in this paper) [36]. The patients were held in sterile environment in separated oxygen tents. For decontamination, co-trimoxazole, vancomycin, tobramycin, amphotericin-B or nystatin and acyclovir was administered according to the standard protocol, which was completed with administration of anti-cytomegalovirus therapy if needed. From clinical samples of patients, taxonomically heterogeneous CNS strains were isolated with coincidence of

Gram-negative potentially pathogenic microorganisms (i.e. *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp.) without manifested bacterial infections.

Detailed analysis of the collected CNS isolates failed to demonstrate uncommon hospital strains [37]. Every patient carried his/her "own" CNS clone divided into subclones by minor differences in their antibiogram. These changes in resistance pattern during hospitalization might be result of acquisition or spontaneous loss of resistance determinants as was reported in the clinical practice [45, 46]. Strains colonizing individual patients differed from each other in biotype, phage pattern, resistance pattern and/or slime production [37].

Using the same statistical analysis, examination periods II and III also failed to reveal a hospital-acquired infection. No predominating endemic CNS strain was demonstrated either in clinical specimens or in environment and the staff.

Although larger accumulations of CNS isolates proved not to be nosocomial epidemics, considering the compromised immunity of patients makes surveillance of sporadic persistence of nosocomial strains reasonable. Occurrence of only one phage pattern of *S. epidermidis*, Ph5/ Ph9/ Ph10/ Ph12/ Ph13/ Ph15/ U4/ U14/ U16/ U20/ U33/ U46, was observed during the four year investigated. Plasmid pattern of these strains showed differences according to the different antibiogram. These results do not establish either maintenance of permanent hospital flora in the environment or role of staff carriage.

Considering the high risk of a potential CNS infection of patients after bone-marrow transplantation the change in disinfection and the accurate keeping of hygienic rules reduced positivity considerably.

In conclusion, typing of coagulase-negative staphylococci may help to clarify connections between hospital isolates and their possible clinical or epidemiological relevance.

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THE EFFECT OF PROSTAGLANDINS ON THE REPLICATION OF ADENOVIRUS WILD TYPES AND TEMPERATURE-SENSITIVE MUTANTS*

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Replication and transformation by adenoviruses involve their interaction with several cellular regulatory mechanisms. Alterations in the phosphorylation of both cellular and viral polypeptides by secondary messenger cAMP and cGMP dependent protein kinases (PK) determining outcome of viral effects may be influenced by external physiological stimuli, among them by prostaglandins (PG). HEp-2 cells infected with representative serotypes of human adenovirus (Ad) groups and two temperature sensitive (*ts*) mutants were treated prior to and late postinfection at permissive (32 °C) and restrictive (39 °C) temperatures by different PGs. PGF_{2α} augmented replication of both oncogenic Ad-12 and latent Ad-1, Ad-5 serotypes as well as Ad-5 mutants at 32 °C and that of mutant *ts18* (defective in pVI phosphorylation) but not that of *ts19* (defective in pX phosphorylation) at 39 °C. PGE₂ was shown to be inhibitory, but the replication of nononcogenic Ad-8 was not affected by PGs. PGI₂ slightly enhanced all types, while indomethacin, inhibitor of endogenous PG synthesis with double unsaturated bonds moderately inhibited replication of all serotypes, except that of Ad-12. It is concluded that augmenting effects by PGF_{2α} during viral entry, cytoplasmic transport and late phase, but not in the early phase of adenovirus replication cycle, are due to enhancement of the non-specific cellular mechanisms, which support mitosis in the uninfected cells. Their activities are controlled by the late viral replication machinery, which process had been programmed in the early interaction of viral and cellular regulatory factors.

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Posttranslational modification of proteins, mainly by phosphorylation, is important in regulating their enzymatic and other functions [1–6]. Viruses contain and induce a limited number of polypeptides. Most of them are phosphorylated on several aminoacids, therefore potentially they are able to fulfil different functions [7, 8]. Both cellular and viral protein kinases (PKs) are able to modify phosphorylation pattern of host's and viral proteins [6, 9, 10]. The greater part of normal phosphorylation is carried out by cyclic nucleotide (CN) dependent PKs, as cyclic adenosine 3',5'-monophosphate (cAMP) dependent PK-A [11–14] or cyclic guanosine 3',5'-monophosphate (cGMP) dependent PK-G [15, 16]. Several CN-independent PKs are also known to act, e.g. PK-C [7, 16, 17]. The substrate specificity of CN dependent PKs is wide, they are able to utilize several structural and non-structural polypeptides of viral origin [18]. Cellular cAMP level is upregulated by prostaglandin E₂ (PGE₂) and partially by the labile PGI₂, while cGMP level is increased by PGF₂ and in a smaller degree by PGA, PGG, PGH [19, 20].

The effects of cAMP–PGE₂ and cGMP–PGF₂ systems are of antagonistic in many aspects [15]. Cyclooxygenase and peroxidase are the key enzymes in the synthesizing pathway of both PGE₂, PGF_{2α} and other intermediates as PGI₂ (prostacyclin). Inhibition of these enzymes by salicylates, indomethacin, etc. decreases endogenous production of both PGE₂ and PGF_{2α} [21–23], but UV-irradiation, stress or bacterial endotoxin (LPS) augment these enzyme activities [24]. PGs are produced and released continuously in different ratio by all cell types, and they influence several functions of other cells as local hormones [19, 21, 25, 26] through secondary messengers cAMP and cGMP by binding cellular receptors [15, 24].

The relationships between PG–CN–PK cascade and virus infections, including immunological and oncological aspects have been recently reviewed extensively by us [27]. Here it is worth to mention important mechanisms in their action, only. In eukaryotes, cAMP enhances cAMP dependent histone kinase, which phosphorylates histones rich in basic lysine (Lys) [28]. This process is a prerequisite to induce cellular enzymes having a role in virus replication and transformation [18]. Acidic nuclear proteins are phosphorylated on serine (Ser) or threonine (Thr) residues by cGMP dependent kinases, in the G₁ and S phases of normal cells, however, it hardly takes place in G₂ phase and mitosis [11, 18]. Finally, these processes result in the modification of histone binding to DNA, therefore revert non-specific translational inhibition by histones [1, 18]. The cAMP level rises in G₁ phase, while it decreases in G₂ phase and it is the lowest during mitosis [1, 11]. The changes of cGMP are of the opposite direction. PGF_{2α} and cGMP cause cell proliferation, lack of contact inhibition and greater membrane fluidity. PGs alter both mobility and expression of cell surface receptors [29], and they stabilize them towards other mediators [24, 30]. cAMP by phosphorylation also stabilizes intracellular microtubuli and membranes, which are important in life cycle of viruses by maintaining cell compartmentalization during their production [19, 24, 27].

Long-term infection by many viruses can result in their persistency in the host. The viral genome is present but infectious particles are not produced, except during intermittent episodes of reactivation from so-called latent state. In order to persist, the virus must regulate its lytic potential and it has to avoid elimination by the host's immune system, which processes different in the case of oncogenic and latent

adenovirus (Ad) serotypes have been recently reviewed [31]. Lytic viruses can persist in vitro under conditions where only a small fraction of total cells are infected at a given time. This "cycling" infection has been implicated in the persistence of human adenovirus in vivo [32] and in vitro [33]. Several types of viral variants such as temperature sensitive (*ts*) mutants, defective interfering particles, etc. have been used in the establishment of persistent infection in vitro [34, 35].

Adenoviruses are good candidates to study the effect of external stimuli on their relationship between viral and cellular regulatory mechanisms. Their infectious cycle includes two programs of gene expression, early (E) and late (L), which are separated by the onset of viral DNA replication. Production of late polypeptides occurs approximately at 20 h postinfection (p.i.) at maximal efficiency [36]. During early phase, seven different transcription units are activated, designated early regions E1A, E1B, E2A, E2B, E3, E4, and late region 1 (L1). Many of the early products are important in normal progression of the viral infectious cycle into its late phase [37]. It has recently been revealed that E1A, E2, E3, E4 regions contain sequences similar to cAMP responsive elements (CRE) of cellular genes. A cellular transcription factor, CRE binding protein (CREB) phosphorylated by PK-A specifically binds to these sequences upregulating their functions [7, 9]. Several types of cellular transcription factors are found in inactive state in mammalian cells [6]. In cooperation with E1A proteins, cAMP induced phosphorylation of members of these activating transcription factor ATF/CREB and AP-1 (jun and fos) families in many cell types has been implicated in the transcriptional activation of E1A inducible other viral genes [9, 10, 38]. Another interactions between E1A proteins and the cAMP signalling system as well as oncogenes play a role in the induction of oncogenic transformation depending on cell types and cell cycle [8, 39-41]. The influence of external factors on the late phase production and assembly of virus constituents of adenovirus replication is less explored. Among 11 structural and limited number of nonstructural polypeptides, the CN independent viral PK phosphorylates either minor structural polypeptides (IIIa, VI, X) [42] or core polypeptides (V, VII), a 72 kD single stranded DNA binding protein (DBP) [43, 44] on Ser/Thr residues, but it is unable to utilize histones. On the other hand, all structural polypeptides of adenoviruses contain residues which can be phosphorylated in vitro by cAMP dependent PK obtained from rabbit reticulocytes or KB cells. The substrate specificity of latter enzymes are wide, they also utilize histones, casein, protamine [42]. Studies on assembly or late *ts* mutants provided further important details on the role of phosphorylation in their life cycle. In the young virion, altered configuration at restrictive temperature (39 °C) inhibits proper phosphorylation by viral PK and cleavage by viral protease on the precursor of pVI, a product of L3 gene in Ad-5 mutant *ts18* [45]. Similarly, at restrictive temperature lack of proper phosphorylation of a 11kD, 79 amino acid residue (R) polypeptide, precursor of polypeptide X encoded by L2 gene together with pVII, pV, pIII virion components [46, 47], inhibits cleavage in Ad-5 *ts19* mutant [45]. pVI molecules stabilize hexon ninemers, while pX in the viral core has a role in tethering DNA [36]. Their damages prevent virus maturation and assembly [45]. Production of these mutants was found diminished even at permissive temperature, in spite of the fact that both pVI and pX could be phosphorylated in vitro by cAMP dependent beef heart PK, but not by virion associated PK at semipermissive temperature (37 °C) [45]. The possible differences in phosphorylation pattern and their biological significance are unknown.

In contrast to other viruses [48, 49 and see references in 27], the effect of prostaglandins (especially $\text{PGF}_{2\alpha}$) and cyclic nucleotides on the cellular entry and transport processes as well as the late phase of adenoviruses with different oncogenic potential has not been explored. Using representative serotypes of different adenovirus groups and two *ts* mutants mentioned above, we studied the effects of both PGE_2 and $\text{PGE}_{2\alpha}$ and the effect of inhibited endogenous PG production on the first encounter between cells and viruses as well as on late functions in virus assembly.

Materials and methods

Viruses and cell cultures. Oncogenic (Group A) adenovirus type 12 (Ad-12, strain Huie, obtained from Dr.G.Berencsi, Budapest, Hungary), nononcogenic (Group D) type 8 (Ad-8, strain K, from Dr.A.Lengyel, Budapest, Hungary), latent (Group C) type 1 (Ad-1, from Dr.U.Krech, Switzerland) and type 5 (Ad-5, from Dr. H.G.Pereira, London, UK) were grown in HEP-2 cultures (obtained from the National Institute of Health, Bethesda, MD, USA) by a standard procedure [50, 51], partially purified [31] and titrated [52] at 37 °C. Two *ts* mutants of Ad-5, viz. *ts18* and *ts19* were obtained from Dr.B.Taródi (Szeged, Hungary) as it has been described [52]. Both mutants were produced in HEP-2 cultures at permissive temperature (32 °C) and titrated at both 32 °C and nonpermissive temperature (39 °C) [52, 53], subsequently 1 tissue culture infectious dose (TCID_{50}) was determined. Taking into account that little amounts of viruses were planned to use throughout the experiments, we preferred to apply this minimal, but surely infectious dose to the usual TCID_{50} . Briefly, virus stocks were serially diluted in tenfold manner, placed on subconfluent HEP-2 cultures in 10 parallel samples. Cytopathic effects in percentage of tubes versus dilution were represented graphically, and extending the straight part of titration curves towards dilution resulted in calculation of TCID_{50} by interpolation [50, 52]. Viruses in 1 ml aliquots were stored at -80 °C and used only once.

Treatment with prostaglandins indomethacin and infection of cell cultures. Concentrations of PGs and indomethacin, which are nontoxic and do not influence cell growth, were determined by mixing compounds diluted serially to both subconfluent monolayers and plating efficiency assays of HEP-2 cells as it has already been described [50]. Quantities below these values were used in the experiments. The effect of PGs and indomethacin on the absorption and transport phase of virus replication was studied by treating cells with compounds prior to infection: 2×10^5 HEP-2 cells in Eagle's MEM containing 2% fetal calf serum (FCS, Phylaxia, Budapest, Hungary) and antibiotics were aliquoted in test tubes. Upon reaching confluency, next day their medium was replaced by fresh one containing tenfold dilutions of $\text{PGF}_{2\alpha}$ (Chinoin, Budapest, Hungary), PGE_2 (Sigma, St.Louis, MO, USA), PGI_2 (Sigma) or indomethacin (Chinoin). Twenty-four hours later, treated and mock-treated cultures were infected with 0.1 ml suspension of 1 to 10 TCID_{50} viruses for synchronized lytic infection and 10^{-1} to 10^{-3} TCID_{50} inocula for latent infection [33, 50]. Influence of compounds on the late phase of virus infections was studied by infecting 24 h old cultures with similar inocula and subsequently treating them at the same concentration of these biological response modifiers between days 1 and 9. All tests were done in 6 parallel samples. Cultures of wild types were incubated at 37 °C, while those infected with Ad-5 WT and its mutants were kept at both 32 °C and 39 °C for several days. In some cases, cell cultures treated and infected at one temperature were shifted to higher or lower temperatures. At different time intervals, 0.1 ml aliquots of supernatants were removed for virus titration as described above.

Results

The effect of PGs and indomethacin on the replication of adenovirus wild types and two mutants are summarized in Table I. Mainly pretreatment with $\text{PGF}_{2\alpha}$ but also late treatment enhanced replication of oncogenic type 12 and latent type 1, but it had no effect on nononcogenic type 8. $\text{PGF}_{2\alpha}$ enhanced replication of Ad-5 WT, and this effect was more pronounced at both permissive (Fig. 1: B, C) and restrictive (Fig. 1: E, F) temperatures, if smaller inocula were used. Results obtained at 37 °C were similar to those at 32 °C (data not shown). Pretreatment and late treatment had similar effects. If larger inocula were used and therefore virus replication was faster, the promoting effect of PGF_2 had no advantage (Fig. 1: A–F). Extremely small inocula of WT did not result in virus replication at any temperature (Fig. 1: A, D), but similar infection followed after $\text{PGF}_{2\alpha}$ treatment of cultures resulted in detectable virus replication (Fig. 1: B, E). This means a possibility for reactivating latent/persistent infection. This effect manifested itself immediately after $\text{PGF}_{2\alpha}$ addition (Fig. 1: C, F).

Table I

The effect of prostaglandins and indomethacin on adenovirus wild types and two mutants

Compound	Concentration	Timing of treatment to infection	Ad-12			Ad-5					
			Ad-12	Ad-8	Ad-1	WT		ts18		ts19	
						32°C	39°C	32°C	39°C	32°C	39°C
$\text{PGF}_{2\alpha}$	0.01–10	Prior	++	0	++	+	0	+++	++	+	0
	µg/ml	Late	+	0	+	++	0	+++	+	0	0
PGE_2	0.01–10	Prior	--	0	--	–	0	–	0	–	0
	µg/ml	Late	--	0	–	–	+	–	0	–	0
PGI_2	1–10	Prior	+	+	+	0	+	+	0	0	0
	µg/ml	Late	+	++	0	0	0	–	0	–	0
Indomethacin	10^{-8} – 10^{-5}	Prior	0	–	--	–	0	–	0	–	0
	M	Late	0	–	--	–	0	–	0	–	0

Quantitation:

- +++ = enhanced replication (2.6–3.5 lg)
- ++ = enhanced replication (1.6–2.5 lg)
- + = enhanced replication (0.6–1.5 lg)
- 0 = no effect
- = diminished replication (0.6–1.5 lg)
- = diminished replication (1.6–2.5 lg)

Replication of mutant ts18 at 32 °C took place at lower efficiency than that of Ad-5 WT (Fig. 1: A and Fig. 2: A), but it reached the level of WT by the effect of $\text{PGF}_{2\alpha}$ (Fig. 1: B, C and Fig. 2: B, C). Enhanced replication of ts18 by $\text{PGF}_{2\alpha}$ pretreatment exceeded that of WT in the cases of higher inocula (Fig. 1: B and Fig. 2: B). No growth of ts18 could be detected at restrictive temperature (Fig. 2: D). Pretreatment of cells with $\text{PGF}_{2\alpha}$ made its limited replication possible (Fig. 2: E).

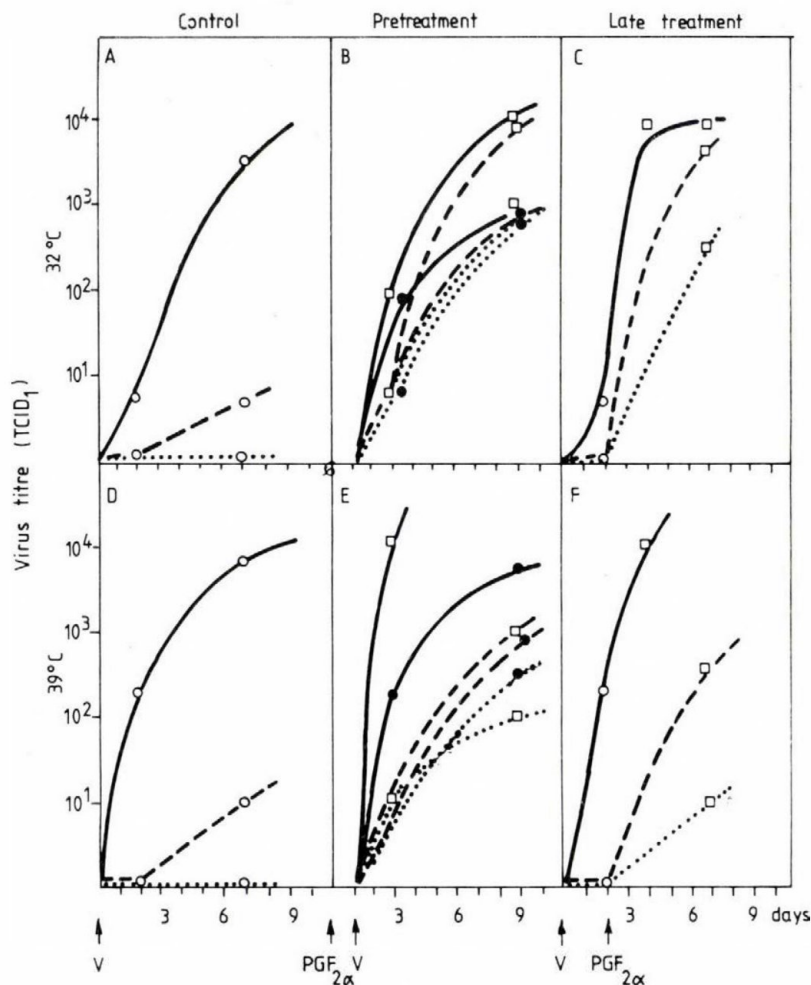


Fig. 1. The effect of PGF_{2α} on the replication of Ad-5 WT. Virus inoculum : ——— 1 TCID₅₀; - - - - - 10⁻¹ TCID₅₀; ····· 10⁻² TCID₅₀. Prostaglandin concentration in the supernatant : □ 10 µg/ml; ● 1 µg/ml; ○ 0 µg/ml

Although its degree depended on the dose of PGF_{2α}, mutant's quantities remained well below levels of wild type at restrictive temperature (Fig. 1: E) or below levels of both WT and *ts18* at permissive temperature (Fig. 1: B and Fig. 2: B). The phenomenon means that PGF_{2α} treated cultures do not harbour latent/persistent infection. Sudden onset of replication by *ts18* was observed if PGF_{2α} was applied 24 or 48 h postinfection (Fig. 2: F). Temperature-shift experiments from 32 °C to 39 °C resulted in the immediate stop of *ts18* replication, while it continued, however, in

lesser extent, if cell cultures were treated with $\text{PGF}_{2\alpha}$. Shifting cultures infected at 39°C by *ts18* to 32°C resulted in immediate onset of replication, but this phenomenon and its enhancement by $\text{PGF}_{2\alpha}$ could not reach the titres of those cultures, which had been kept continuously at 32°C (data not shown). Mutant *ts18* exhibited cytopathic effect similar to that of WT (Fig. 3).

The effect of $\text{PGF}_{2\alpha}$ on mutant *ts19* showed completely different pattern (Table I). Its replication after infecting cells with large inocula was not augmented by $\text{PGF}_{2\alpha}$.

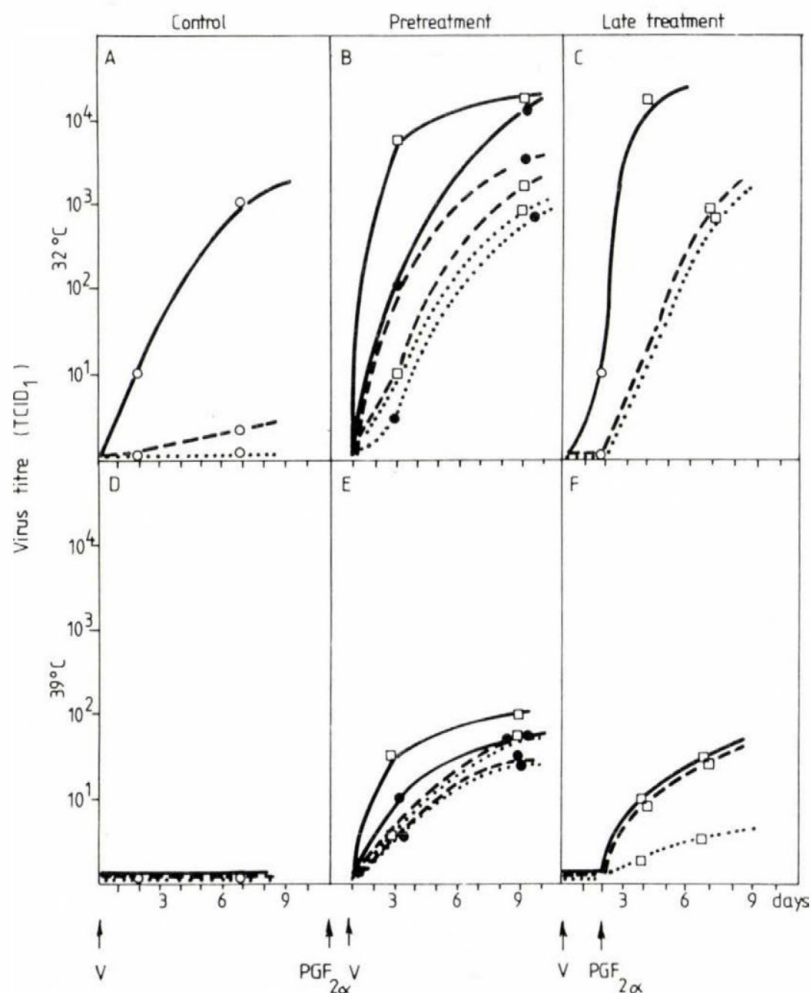


Fig. 2. The effect of $\text{PGF}_{2\alpha}$ on the replication of Ad-5 *ts18* mutant. Virus inoculum : ——— 1 TCID_{50} ; - - - - - 10^{-1} TCID_{50} ; 10^{-2} TCID_{50} . Prostaglandin concentration in the supernatant : $\square$ 10 $\mu\text{g/ml}$; $\bullet$ 1 $\mu\text{g/ml}$; $\circ$ 0 $\mu\text{g/ml}$

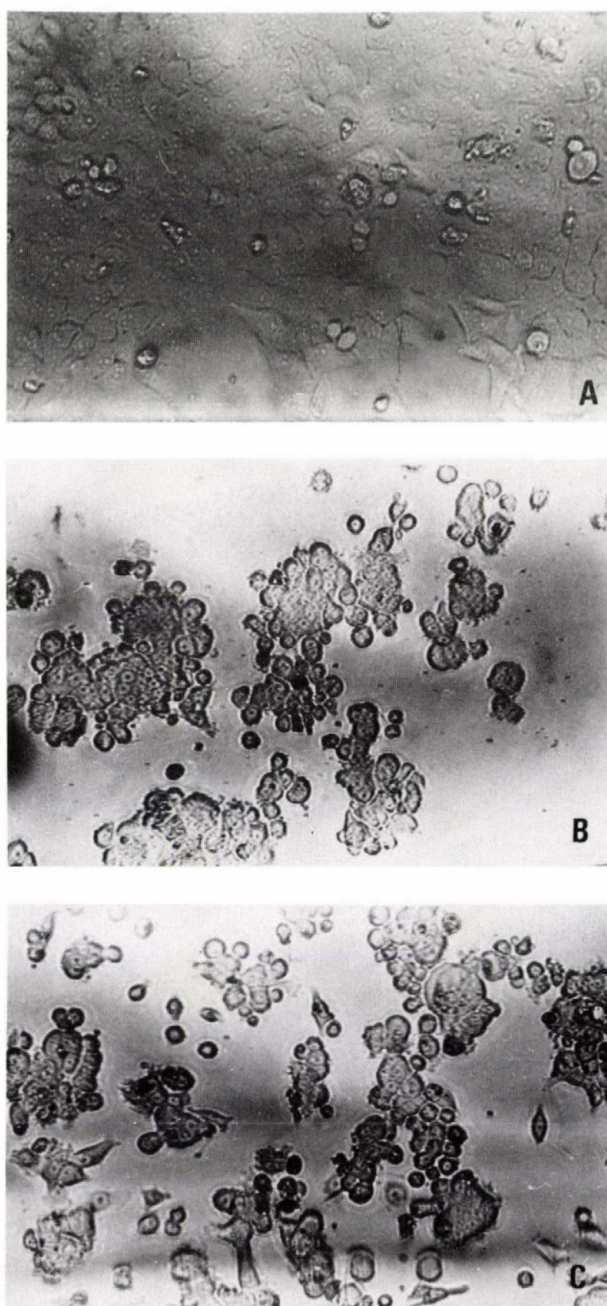


Fig. 3. 72 h old uninfected HEP-2 cultures (A) are confluent. Both Ad-5 WT (B) and PGF₂ α -activated ts18 mutant (C) result in clustering of swollen, rounded cells. $\times 250$

At permissive temperature (Fig. 4: A–C) its replication after infecting HEp-2 cultures with small inocula (e.g., 10^{-1} TCID₅₀) although enhanced (Fig. 4: B, C), but remained below augmented replication of both WT (Fig. 1: B, C) and *ts18* (Fig. 2: B, C). Mutant *ts19* does not replicate at 39 °C (Fig. 4: D) and PGF₂ was unable to activate it (Fig. 4: E, F).

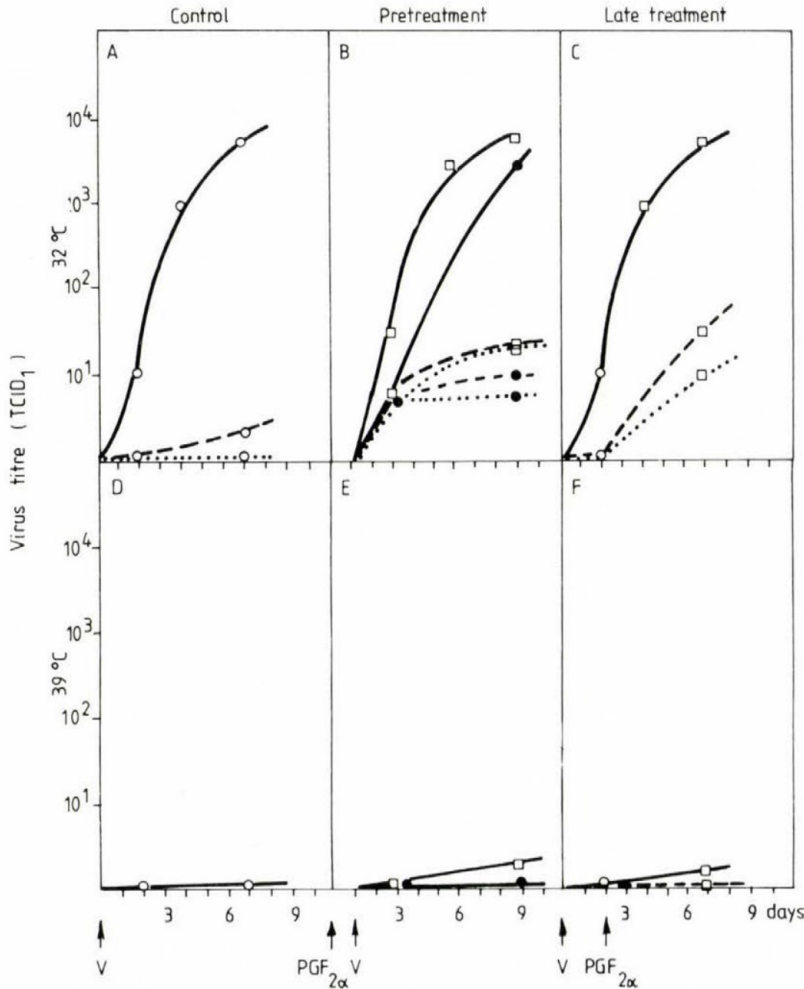


Fig. 4. The effect of PGF_{2α} on the replication of Ad-5 *ts19* mutant. Virus inoculum: ——— 1 TCID₅₀; - - - - - 10⁻¹ TCID₅₀; ······ 10⁻² TCID₅₀. Prostaglandin concentration in the supernatant: □ 10 µg/ml; ● 1 µg/ml; ○ 0 µg/ml

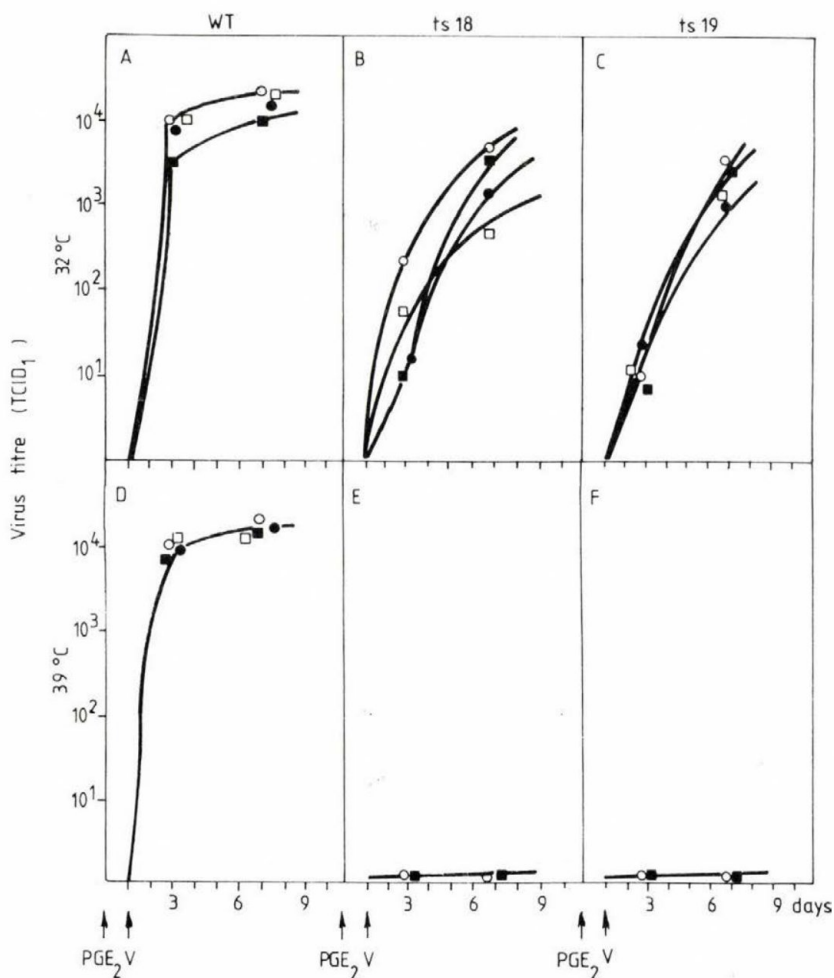


Fig. 5. The effect of PGE₂ on the replication of Ad-5 WT and its mutants. Virus inoculum: — 1 TCID₅₀. Prostaglandin concentration in the supernatant: □ 10 µg/ml; ● 1 µg/ml; ■ 0.1 µg/ml; ○ 0 µg/ml

PGE₂ applied before or after infection decreased replication of Ad-12 in a similar ratio (Table I). Pretreatment of Ad-1 infected cultures resulted in a somewhat stronger inhibition than late treatment did. PGE₂ did not influence growth of Ad-8.

Replications of Ad-5 WT and mutant *ts19* were moderately inhibited (Fig. 5: A, C), but that of *ts18* was affected stronger and in a dose-dependent manner (Fig. 5: B). At restrictive temperature PGE₂ did not alter growth pattern of Ad-5 WT (Fig. 5: D) and had no effect on mutants (Fig. 5: E, F). Late treatment with PGE₂ of infected cells was nearly identical to pretreated cells (data not shown).

Pre- or late treatment of HEP-2 cells with PGI₂ slightly enhanced replication of Ad-12. Pretreatment exerted stronger increase on Ad-8 growth than late treatment did. Only pretreatment of cells with prostacyclin augmented moderately latent type Ad-1 and Ad-5 WT at elevated temperature, as well as its mutant *ts18* at permissive temperature. Mutant *ts19* was not affected. Late treatment of infected cells slightly inhibited replication of both mutants at 32 °C (Table I).

Indomethacin had no effect on Ad-12 and Ad-5 WT as well as its mutants at restrictive temperature, while both pre- or late treatments of infected cultures definitely diminished production of nononcogenic Ad-8 and latent types Ad-1 and Ad-5 including its mutants (Table I).

Discussion

These observations show that during initial steps of infections and in their late phase the sensitivity of latent and oncogenic types of adenoviruses is similar concerning the enhancing or diminishing effects of PG derivatives, however, difference is found in the magnitude of their response. Nononcogenic adenovirus is less affected.

The augmented virus titres in PGF_{2α} treated cells can be the consequence of multiple effects. PGF_{2α} induced cGMP renders cell membranes and microtubules flexible, which might facilitate formation of endocytic vesicles for virus absorption and particle transport across the cytoplasm in the process that involves microtubules. The antimitotic agent vinblastine by reorganizing microtubules into a concentrated mass reduces the amount of virion derived DNA that associates with nuclei by 2 h after penetration, consequently causing delay in the one step growth cycle of the virus [54]. Another factor which suggests facilitation of adenovirus uptake is that the majority of cGMP synthesizing guanylate cyclase molecules, stimulated by PGF_{2α} is localized in the cell membrane and cytoplasm, while adenylate cyclase stimulated by PGE₂ is dominating in the nucleus [24], where transcriptional regulation of early adenovirus genes takes place. Reaching the nuclear pores, viral core enters nucleus, and DNA plus polypeptides V, VII, μ and terminal polypeptide (TP) are converted into a viral DNA-cell histone complex that is chromatin like [36]. This seems to be advantageous for interacting with cellular regulatory mechanisms.

Recent observations suggest that there is a functional interaction between E1A proteins and cAMP signalling system and this interaction may play a role in the induction of cellular transformation [39]. There are two major E1A protein species of 243 and 289 aminoacid residues (R) encoded by the alternatively spliced 12S and 13S E1A mRNAs, respectively. These proteins are highly related, differing only by an internal 46R region that is unique to the 289R protein. Activation of viral gene

transcription is encoded primarily within the 46R region [55], while the 243R protein which lacks this region and is a poor activator of viral transcription, is able to activate several cellular genes [39] interfering with normal cell cycle controls [41]. These processes can lead to transformation. 243R disrupts cellular E2F transcription complexes, including those forms containing the 105kD protein product of the retinoblastoma growth suppressor gene [6, 40], cyclin-histone H1 kinase and other Ser/Thr kinases [40, 41], then E2F molecules can bind to promoters of such genes that are required for DNA synthesis and consequently let cellular transition from G₁ to S phase [6, 38–41]. The level of cAMP is found high in G₁ and S phase, but it decreases in G₂ phase and is the lowest in mitosis. Studies on several viruses show that transformation depends on a process with decreasing cAMP level [18, 27, 56]. The fate of cells, namely, to undergo transformation or to support virus production seems to be determined during a cAMP sensitive period of interacting adenovirus early gene products and cellular factors. Similar observations on transforming gene products of both DNA viruses [57, 58] and RNA tumour viruses [59] show that several of their gene products can complement or substitute each other. These processes depend on the host's nature and its state of differentiation. Undifferentiated F9 teratocarcinoma cells could complement early transcriptional defect of the E1A deletion (*dI*) mutant *dI312*, however, after differentiation with retinoic acid and cAMP the cells lost their complementing activity [36]. Adding dibutyryl- (db) cAMP to infected fibroblastic cell lines (BHK-21, HEK, WI-38) early, resulted in almost total inhibition of replication by Ad-12 and a considerable inhibition of Ad-2, but neither of them was inhibited in tumour derived human cell lines HeLa and KB [60]. HeLa cells constitutively express E7 and E6 proteins of human papillomavirus HPV type 18 [61]. E7 has some of the transactivating characteristics of adenovirus E1A proteins [62], and therefore its enhancement during expression of early adenovirus genes can mask inhibitory effects during entry and transport processes.

In our experiments, PGF_{2α} readily activated adenovirus serotypes 12, 5, 1, if they remained latent after infecting cells with small inocula. This corresponds to the fact that induction of host transcription factors appears to be independent of new protein synthesis but results from posttranscriptional modification of existing host factors [36].

Assembly of both oncogenic and latent adenovirus types was augmented by PGF_{2α} treatment. In the young virion, the localization of pVI molecules around hexon ninemers is partially external [42]. This might make precursor of pVI sterically accessible to a cGMP activated cellular PK. Young virions are open and their structure is less tight [36], which gives another possibility for a cellular PK to phosphorylate, then viral protease can cleave that protein. The viral PK is not thermosensitive [42], but assembly of normal polypeptides is drastically inhibited at 42 °C, which is reversible upon temperature shift to 37 °C [36]. In general, overproduction of viral components is accompanied by low efficiency assembly [36], and PGF_{2α} seems to improve this process.

The 11 kD precursor of polypeptide X [46] is located in the more compact core [36], that might be less accessible to these enzymes. Virion associated proteinase, a 23kD polypeptide coded by L3 gene [36], has a role in cleaving polypeptides VI, VII, VIII [36, 45, 46] and 11kD [46]. Studies on several serotypes revealed that both the

proteinases and the substrate cleavage sites are highly conserved among human adenoviruses [47], but the aminoacid sequences surrounding the cleavage sites in pVI, pVII and 11kD polypeptides are slightly different [46]. Selective cleavage by this enzyme is not unique: in Ad-2 *ts3* mutant, cleavage of pVII is blocked, but cleavage of pVI precursor (designated Va 27kD protein) to 24 kD and pVIII (designated Vb 24kD) to 13 kD are cleaved normally [63]. Additional cleavage of pX generates approximately 4kD polypeptide μ [46] found in the core of mature particles [36, 46]. While core polypeptides V and VII are basic, rich in Arg, like histones [36], 11kD and μ polypeptides are extremely basic with high percentages of Arg, His, but without Lys, Asp, Try, Tyr and acidic residues aspartic acid and glutamic acid. A computer search of the protein sequence data has not revealed highly significant homologies with other proteins, although the central segment resembled protamines [46]. The lack of Tyr in its structure renders polypeptide μ resistant to tyrosine PKs, which latter are important in tumourigenesis by many oncogenic viruses [27]. It is postulated that the unique structure might give polypeptide μ a key role in both regulating integration of adenovirus genome into host chromosomes with converting core polypeptides to histones [36] and in the last steps of virus maturation [36] leading to virus assembly and release irreversibly. Another example that a minor polypeptide has an important regulatory role is that in vesicular stomatitis virus, the state of phosphorylation affected by *ts* mutations of minor polypeptide NS, influences its regulatory role by binding to the large (L) polypeptide having the transcriptase activity [64].

The molecular mechanism of the onset of the late gene expression heralded by the replication of the viral DNA template is not understood [37]. Host DNA and protein syntheses are gradually inhibited by that time. When viral DNA synthesis was programmed to start during the G₁ phase of the cell cycle, no subsequent host cell DNA synthesis occurred in the following S period. However, if adenovirus DNA synthesis in synchronized cells coincided with the S phase, host DNA synthesis remained normal for many hours. Adenovirus infection inhibited initiation of host cell DNA synthesis but not its maintenance [36]. Transcription of many early genes is inhibited late, but the 72kD DBP, phosphoprotein product of E2 gene [44] is expressed late and suppresses several early functions. Abortive infections leading often to cellular immortalization show no control of E genes with low level DBP [65]. E4 products are important for nuclear accumulation and stability of primary transcripts of viral late genes, especially that of pVI, and they do not affect the stability of host sequences [37]. Although primary transcription of host sequences is still active in the nucleus, there is a failure of transporting cell mRNA precursors to the cytoplasm. Virus associated RNAs bind to a 68kD cellular PK, block its autophosphorylation and thereby inhibit the subsequent inactivation of protein initiating factor eIF2 α and inhibit host protein synthesis [36]. The inhibition of cell DNA synthesis might then be secondary to the shut-off of host protein synthesis; consequently very few of the cells infected at high multiplicity divide [36].

cGMP level rises during G₂ phase and it is high during mitosis, cGMP is regarded as a positive signal for normal cell division (see ref. in [27]). It is reasonable to assume, if virus replication is programmed during the early virus-host cell interaction, instead of supporting normal cell division, cGMP dependent aspecific mechanisms support basically similar production of viral structure polypeptides and

DNA. It is generally accepted that protein substrates behave similarly in respect of phosphorylation in subsequent enzyme reactions [3, 14].

Since the first presentation of our observations [66] still few data have been available on studying effects of $\text{PGF}_{2\alpha}$ on viruses. $\text{PGF}_{2\alpha}$ was shown to shift life cycle of murine mammary tumour virus to production by cell cultures [67]. Both $\text{PGF}_{2\alpha}$ and serum, the source of $\text{PGF}_{2\alpha}$ stimulated HSV-2 replication [68], while db-cGMP treatment of HEp-2 cells increased yield of HSV-1 [49]. Intracellular cGMP level is increased by mitogens [69] that are required for efficient replication by many viruses in otherwise nonpermissive or semipermissive cell types [70]. cGMP stimulated measles virus replication and its cytopathic effect, as well as it markedly increased infectious particle production, if cells were stimulated at 48–72 h postinfection. Only limited cell division occurred after infection [48].

Life cycle of nononcogenic adenovirus does not involve intensive interaction between viral and cellular regulatory mechanisms, and this could account for its insensitivity to PGE_2 or PGF_2 mediated effects. PGI_2 exhibited slight enhancing effect on the replication of all adenovirus serotypes tested. This may be relevant to the fact that exogenous PGI_2 seems to function as antitumour agent inhibiting metastasis occurrence [71]. Antitumour effects by interferon (IFN) and PGI_2 are synergic [72], but adenoviruses do not exhibit significant sensitivity to IFN [36]. Inhibition of the endogenous PG synthesis in HEp-2 cells by indomethacin had no effect of the replication of Ad-12. Transformation of 3Y1 cells by Ad-12 did not affect endogenous cAMP level, either [73]. Slight decreasing effect of indomethacin on the replication of latent and nononcogenic serotypes might be the consequence of its general inhibitory effect on PGs with double unsaturated bonds [21, 22], while unbalanced presence of PG analogues with single unsaturated bond can disturb the replication machinery of adenoviruses. Inhibiting production of several viruses by indomethacin has already been shown [27].

Here we studied the direct effect of PGs on adenoviruses. PGs and related compounds are known to modify immune functions, which are important in eliminating virus particles and infected as well as transformed cells [74, ref. in 27 and 31]. On the other hand, several adenovirus early gene products discussed above by interacting with cellular regulatory mechanisms result in avoiding immune surveillance (reviewed recently in [31]). All these factors taken together imply that outcome of adenovirus infection might be influenced by biological response modifiers [75].

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EFFECTIVENESS OF ANTIBIOTICS ON THE AUTOCHTHONOUS *ESCHERICHIA COLI* OF MICE IN THE INTESTINAL BIOFILM VERSUS ITS PLANKTONIC PHASE

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The effectiveness of antibiotics was tested on the autochthonous *Escherichia coli* in biofilm mode of growth in large bowel pieces as well as on the predominant faecal *E. coli* isolated from the same SPF mice in planktonic phase of growth. Aminoglycoside antibiotics, chloramphenicol, oxytetracycline, erythromycin and lincomycin-clindamycin treatment had a very limited effect in the intestinal biofilm. Surprising ineffectiveness was found with polymyxins: polymyxin B showed a Minimal Bactericidal Dose of 0.78 µg in planktonic phase, while it was 400 µg for *E. coli* incorporated in the biofilm matrix. In contrast to the above groups of antibiotics, the beta-lactam drugs were effective both in the biofilm and in the planktonic phase growth of *E. coli* and their derivatives with broad or broader spectrum exerted an increased biofilm activity. Polymyxin B showed no sign of penetration into the colonic mucus, but on the other side ampicillin concentrated about three–four times in the intestinal matrix.

The biofilm mode of growth, which is characterized by a slow growing tendency in an environment with limited nutritional (O₂) capacity, adhesion to surfaces, production of an exopolysaccharide matrix is widespread in Nature [1]. Biofilms of medical interest are the normal flora of the body, some pathological processes (e.g. cystic fibrosis, osteomyelitis, etc.), and biofilm production on the surfaces of medical devices (canules, drain tubes, catheters, pacemakers, etc.). The exopolysaccharide matrix provides for the bacterial, fungal members of the biofilm a well-pronounced defence against antibodies, phagocytic cells, biocids, antibacterial drugs [1]. In our previous work [2] we observed a reduced effectiveness of some antibiotics and disinfectants in a mouse model against the intestinal autochthonous *E. coli* population and against the artificial monoflora of *E. coli*. In this paper we are presenting results on a larger scale of antibiotics tested in this respect.

Materials and methods

Antibiotics applied are listed in Table I.

Media. Luria broth (LB) and LB-agar were used. For counting the colony forming units (c.f.u.) of *E. coli*, Endo agar was applied.

Strains. Testing the level of ampicillin and polymyxin B in the colonic mucus standard sensitive strains obtained from the Hungarian National Collection of Medical Bacteria, Budapest, namely *Staphylococcus aureus* HNCMB 112002 and *E. coli* HNCMB 35034, respectively, were used.

Mice. Outbred SPF mice (LATI, Gödöllő, Hungary) were used in all experiments. For each level of drug concentration 4–4 mice were used.

Testing the effectiveness of antibiotics against E. coli population in the large bowel biofilm. The mice were sacrificed, and about 3 cm long pieces of their colon starting from the cecum were removed. The pieces were cut longitudinally and after a superficial removal of the faecal content, they were put into tubes containing serial 5-fold dilutions of the drug studied. Repeating this test 2-fold dilutions were applied. After three hours of incubation at 37 °C the pieces of colon were transferred into 10 ml portions of LB, shaken vigorously in a Vortex apparatus (CSAV T5001/II, 30 W) for 1 min. Thereafter the mucinous samples were diluted and plated. After overnight incubation at 37 °C the colonies of surviving bacteria were counted and the MBCD value (MBCD – Minimal Bactericidal Dose) determined. The MBCD means the drug concentration killing all bacteria.

E. coli colonies that grew on LB-agar plated from the tube with the highest concentration of the drug served for the test of *E. coli* in the planktonic phase. This isolate was supposed to represent the dominant or only *E. coli* (SPF mice) intestinal population.

Testing the effectiveness of antibiotics against E. coli in planktonic phase. The above isolates made from drug survivors were inoculated into LB and incubated overnight at 37 °C. Serial 2-fold dilutions of antibiotics in 2 ml volume of LB inoculated with 0.05 ml of the above culture, incubated for 3 h at 37 °C and thereafter 0.5 ml aliquots from each tube were plated to determine the in vitro MBCD values.

Testing the level of antibiotics in the mouse colonic mucus. Starting from the cecum, 3 cm long pieces of colon were removed from groups of sacrificed mice each containing 4 animals. The pieces were longitudinal cut and their faecal content superficially removed. After weight determination the pooled colonic pieces were put into 4 ml of saline containing 2 MBCD of ampicillin (8 µg/ml), or 2 MBCD (800 µg/ml) of polymyxin B. After incubation at 37 °C for 3 h, the pieces were rinsed with saline and placed in saline in a ratio of g/4 ml. After one minute of Vortex treating the liberated mucous fluid was separated and 1 ml of chloroform added. After the evaporation of chloroform, serial twofold dilutions of the colonic mucus were inoculated with the standard strain. After incubation at 37 °C for 5 h the MIC (Minimal Inhibitory Concentration) and the MBCD values were determined.

Results

At first, representatives of aminoglycoside antibiotics were tested: streptomycin, kanamycin, gentamicin and spectinomycin. The results are summarized in Table II.

The data show clearly the highly reduced effectiveness of this group of drugs against *E. coli* population in the intestinal biofilm matrix.

Table I

List of Antibiotics

ANTIBIOTICS	Trade name	produced by
Streptomycin	Streptomycin	Medexport/USSR
Kanamycin	Kanamycin	Medexport/USSR
Neomycin	Mycerin	Medexport/USSR
Gentamicin	Gentamycin	Chinoi/Hungary
Spectinomycin	Trobicin	Upjohn/Belgium
Chloramphenicol	Chlorocid	EGIS/Hungary
Oxytetracycline	Tetran pulvis	Chinoi/Hungary
Lincomycin	Lincomycin	Medexport/USSR
Clindamycin	Dalacin-C	Upjohn/Belgium
Erythromycin	Ilotycin	Lilly/USA
Polymyxin B	Polymyxin B	Pfizer/France
Polymyxin M	Polymyxin M	Medexport/USSR
Penicillin G	Penicillin G	Biogal/Hungary
Penicillin V	Penicillin V	Biogal/Hungary
Oxacillin	Oxacillin	Biogal/Hungary
Ampicillin	Ampicillin	Galenika/Yugoslavia
Carbenicillin	Carbenicillin	POLFA/Poland
Cefalexin	Pyassan	Chinoi/Hungary
Cefuroxime	Zinacef	Glaxo/UK
Ceftriaxone	Rocephin	EGIS/Hungary

Table II

Effectiveness of aminoglycoside antibiotics on autochthonous E. coli in the mouse intestinal biofilm and its planktonic phase isolates

Aminoglycoside		Representative drug	MBCD*		ratio sessile:planktonic
group	subgroups		sessile	planktonic	
Streptidine		Streptomycin	800	40	20:1
2-deoxy- streptamines	4,5-substitutions	Neomycin	500	37.5	13:1
	4,6-substitutions	Kanamycin	800	18.2	44:1
		Gentamicin	100	9.3	11:1
Aminocyclitols		Spectinomycin	40	5.0	8:1

* MBCD = Minimal Bactericidal Dose – the concentration in µg/ml killing all bacteria during incubation at 37 °C for 3 h

For testing in the intestinal biofilm and on the planktonic phase of *E. coli* – see Materials and Methods

Other antibiotics, as chloramphenicol, oxytetracycline, erythromycin, lincomycin, clindamycin and the polymyxins had a similarly marked reduction of effectiveness in the biofilm mode of growth of *E. coli* (Table III). In most cases the ratio of effective concentrations between biofilm mode of growth versus planktonic phase of *E. coli* was comparable with the aminoglycosides. Polymyxin B seems surprisingly ineffective in this respect, apparently it has practically no penetration ability into the intestinal biofilm.

Table III

Effectiveness of chloramphenicol, tetracyclines, macrolides, the lincomycin group and polymyxines on autochthonous E. coli in the mouse intestinal biofilm and on its planktonic phase isolates

Antibiotic group	Representative drug	MBCD*		Ratio sessile:planktonic
		sessile	planktonic	
Tetracyclines	Chloramphenicol	1000	80	12.5:1
	Oxytetracycline	100	6.2	16:1
Macrolides	Erythromycin	160	12.5	12.8:1
Lincomycin	Lincomycin	600	37.5	13:1
group	Clindamycin	300	37.5	8:1
	Polymyxin B	400	0.78	512:1
Polymyxines	Polymyxin M	80	3.12	25.6:1

* MBCD=Minimal Bactericidal Dose – the concentration in µg/ml all bacteria during incubation at 37 °C for 3h

For testing in the intestinal biofilm and on the planktonic phase of *E. coli* – see Materials and Methods

In contrast to the above drugs, the beta-lactam antibiotics behaved differently (Table IV): the penicillins, as well as the cephalosporins were effective both in planktonic and biofilm mode of growth of *E. coli*. Some of them, especially the semisynthetic beta-lactam drugs with broad or broader spectrum exerted a seemingly higher activity in the intestinal matrix, suggesting that a concentration may occur in the intestinal mucus.

To test this working hypothesis we determined the level of the ampicillin in the mouse colon mucus exposing it 2 MBCD (8 µg/ml) dose of the drug. The same test was carried out with polymyxin B, exposing the mouse large bowel also to 2 MBCD (800 µg/ml) of the drug. The results are presented in Table V.

Table IV

Effectiveness of the β -lactam antibiotics on the autochthonous *E. coli* in the mouse intestinal biofilm and on its planktonic phase isolates

Antibiotic group	Character	Representative drug	MBCD*		Ratio sessile:planktonic
			sessile	planktonic	
Penicillins	natural	Penicillin G	16	20	1:1
	penicillins	Penicillin V	< 50	> 50	1:1
	lactamase ^R	Oxacillin	80	160	1:2
	broad	Ampicillin	4	12	1:4
	spectrum	Carbenicillin	25	12.5	1:2
Cephalosporins	1st gen.	Cefalexin	4	40	1:10
	2nd gen.	Cefuroxime	0.125	25.6	1:200
	3rd gen.	Ceftriaxone	0.25	1.56	1:6

* MBCD = Minimal Bactericidal Dose – the concentration in $\mu\text{g/ml}$ killing all bacteria during incubation at 37 °C for 3 h

For testing in the intestinal biofilm and on the planktonic phase of *E. coli* – see Materials and Methods

Table V

Uptake of polymyxin B and ampicillin in the colonic mucus of mice exposed to 2 intestinal MBCD

	Polymyxin B	Ampicillin
Total quantity of drugs used for exposition		
colonic exposition	2304 μg	27.5 μg
Dilutions of the colonic mucus giving		
MIC	< 1:2	1:256
MBCD	< 1.2	1:512
The calculated quantity of drugs taken up		
by the colonic mucus	< 0.62 μg	81.9 μg
Percentage ratio of the drug taken up		
by the colonic mucus	< 0.05%	356%
Test strains	<i>E. coli</i> HNCMB 35034	<i>S. aureus</i> HNCMB 112002
Values determined by parallel tests		
MIC	0.16 $\mu\text{g/ml}$	< 0.16 $\mu\text{g/ml}$
MBCD	0.62 $\mu\text{g/ml}$	0.16 $\mu\text{g/ml}$

Legend: MIC = Minimal Inhibitory Concentration read after incubation at 37 °C for 3 h

MBCD = Minimal Bactericidal Dose (no surviving colonies after incubation at 37 °C for 3 h)

For testing of the quantity of drugs taken up by the colonic mucus – see Materials and Methods

In the case of polymyxin B penetration test, the absolute quantity of the drug was 2304 µg for exposition. The colonic mucus neither inhibited nor killed the test strain even in the lowest dilution (1:2). In the parallel test the MBCD value of polymyxin B was 0.62 µg. Consequently, the drug content of the mucus was lower than this value. This means that less than 0.05% of the drug was present in the intestinal mucus. Accordingly, polymyxin B does not penetrate into the matrix and its ineffectiveness on sessile *E. coli* is satisfactorily explained.

On the other hand, the absolute quantity of ampicillin used for exposition was 27.5 µg. The mucus diluted to 1:512 showed an equivalent level of MBCD to the parallel determination of sensitivity of 0.16 µg, therefore the mucus pro ml uptake seems to be of 81.9 µg. This means a concentration of ampicillin to 3.56 times (about 3–4 times) in the colonic mucus.

Taking into account the errors of the biological measurements, this also seems to be a satisfactory explanation that ampicillin had a four times higher effectiveness in the intestinal matrix than in the planktonic phase of the mouse *E. coli*.

Discussion

The results presented give an account of the effectiveness of different groups of antibiotics on the *E. coli* population of the mouse colonic biofilm. Because the animals were SPF mice, the isolated and cultivated dominant *E. coli* represents certainly the planktonic phase of the same *E. coli* population.

Using the vortex treatment, a very intensive shaking to liberate bacteria from the biofilm matrix proved to be satisfactory in our previous experiments [2]. Costerton et al. [4] also applied a slight ultrasonic treatment, but according to our experiments this does not modify the germ yield significantly.

The intestinal biofilm as a defence [3–5] is somewhat special, because the bowel is covered by a thick (about 400 µm) mucus layer [6] and according to Freter et al. [7] most of the intestinal bacteria inhabit the mucus itself. Our previous work in this respect [8], in agreement with their opinion, demonstrated a ligand-receptor relation between different enteric bacteria and the hog gastric mucin, chosen as a model substance. Furthermore, the bacterial ligand proved to be a common outer membrane protein.

The data presented clearly show that aminoglycosides, chloramphenicol, oxytetracycline, erythromycin, lincomycin-clindamycin and polymyxins are practically ineffective, or have a reduced effectiveness on sessile *E. coli*. In contrast, most of them had a satisfactory killing efficacy on *E. coli* in planktonic growth. On the other side, the beta-lactam group of drugs were effective both on sessile and planktonic *E. coli*, and the broad spectrum members of beta-lactams even concentrate in the colonic mucus.

According to Costerton et al. [1] bacteria growing in a biofilm "are embedded in a thick, highly hydrated anionic matrix that conditions the environment of individual cells and constitutes a different solute phase from the bulk of fluids of the system". The extracellular matrix produced by different microbes makes up 73–98% of the biofilm [9]. The biofilm therefore could increase the concentration of a drug, and on the other

hand, the numerous charged binding sites on the matrix polymers could give a marked protection against antibiotics. Another possibility considering this protection may originate from the phenotypic changes of bacteria involving cell envelope protein alterations, or enzymatic changes [1, 10, 11]. The former mechanism was proved to exist in our cases concerning polymyxin B and ampicillin.

The behaviour of biofilm mode of growth points also to some practical consequences:

(i) the routine test of antibiotic sensitivity carried out on planktonic bacteria may be misleading, if a possible biofilm locus is not taken into consideration. The therapy based on sensitivity testing may be effective against planktonic cells causing clinical symptoms, but the elimination of the sessile bacteria is rarely achieved.

(ii) Our antibacterial drugs, including the new ones are tested and selected also on planktonic cultures. Therefore it is an urgent need to test them also for their biofilm matrix effectiveness. Such efforts are in progress using a wide scale of methods [1, 12].

The experimental results presented in this paper are restricted to an intestinal mouse model and to *E. coli*. Since matrix structures may vary with different agents, there is a need to extend such investigations also to other bacteria, especially to *Pseudomonas aeruginosa* and *Staphylococcus* species.

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CHANGES IN THE COMPOSITION AND PEROXIDATION OF YEAST MEMBRANE LIPIDS DURING ETHANOL STRESS

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Lipid peroxidation in *Saccharomyces cerevisiae* cells increased with ethanol treatment of the cells. Such cells have decreased amount of total lipid, phospholipid and free sterols. Sterol:phospholipid ratio decreased slightly in the cells treated with 4% ethanol. However, this ratio significantly increased with further rise in ethanol concentration to 12%. Relative content of glycolipids (glycolipid to phospholipid ratio) increased in ethanol-treated yeast cells. Diphosphatidylglycerol content increased significantly and phosphatidylcholine to phosphatidylethanolamine ratio decreased in ethanol-treated cells. The amount of α -tocopherol decreased during ethanol stress. Catalase failed to counter the effect of ethanol. The results from the present study indicated that ethanol might be interfering with the antioxidant defence mechanisms of the yeast cells.

Besides being an important constituent of pharmaceutical and brewing industries, the emergence of alcohol as a possible biofuel has further widened the interest in its production. Fermentation efficiency of all the ethanol producing organisms declines as ethanol accumulates in the surrounding medium [1]. Ethanol creates a sort of environmental stress. The most resistant strains of yeast have been reported to withstand ethanol concentration up to 8% (w/v) only [2]. Plasma membrane is the site controlling the transport of nutrients into the cell and excretion of various products including alcohol into the surrounding medium. Thus, it is the first sensitive organelle to make contact with ethanol. Alcohols have been reported to alter the chemical composition of the microbial plasma membrane [3]. Alcohol-treated cells are known to have more unsaturation of their membrane phospholipids, thereby increasing membrane lipid fluidity [4]. Ethanol also causes peroxidation of the membrane lipids besides increasing fluidity of plasma membrane in animal cells [5]. The present study is an attempt to correlate alcohol induced membrane lipid peroxidation and composition in *Saccharomyces cerevisiae* var. *ellipsoides*. A possible role of catalase during ethanol stress has also been discussed.

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Materials and methods

Chemicals. Ergosterol, digitonin and phospholipid standards were procured from M/s Sigma Chemical Co., USA. 2-Thiobarbituric acid and α, α' -bipyridyl were purchased from M/s Loba Chemie and all other chemicals were of analytical grade and obtained from local commercial sources. *S. cerevisiae* strain was obtained from the University of California, Davis, USA.

Growth of yeast cells. Yeast cells were cultured in 500 ml Erlenmeyer flasks containing 100 ml synthetic growth medium [6] at $27 \pm 1^\circ \text{C}$ for 24 h. The composition of the medium per litre was glucose 20 g, yeast extract 1 g, MgSO_4 0.5 g, $(\text{NH}_4)_2\text{SO}_4$ 1 g, KH_2PO_4 0.875 g, K_2HPO_4 0.125 g, NaCl 0.1 g and CaCl_2 0.1 g. After the desired period of incubation, the cells were harvested by centrifugation at 5000 g for 15 min. The cells were washed with phosphate buffer (0.01 M, pH 7.0) to remove any adhering metabolite and ingredient of the medium.

To study the effect of alcohol, yeast cells (5 mg dry wt/ml) were incubated for 12 h in 0.01 M phosphate buffer, pH 7.0, containing different concentrations of ethanol and 0.5% dextrose. The cells were shaken on the rotary shaker at a speed of 250 rpm. The cells were harvested, washed and used for various biochemical analyses/estimations.

Malondialdehyde (MDA) assay. Malondialdehyde was estimated by the method of Buege and Aust [7]. Homogenates prepared in chilled 0.9% saline were used for MDA quantification. Two ml of homogenate was taken in a test tube and mixed thoroughly with 4 ml of thiobarbituric acid reagent (15% TCA and 0.375% TBA in 0.25 N HCl). The tubes were placed in a boiling water bath for 15 min. After cooling, the contents were centrifuged and the absorbance was recorded at 535 nm in 1 cm cell against a blank prepared in the same way as test except by taking saline in place of homogenate. MDA content was calculated using the equation:

$$\text{MDA (nm)} = \frac{V \times \text{OD}_{535}}{0.156}$$

where V = total volume of test solution; OD_{535} = absorbance at 535 nm; ϵ = molar extinction coefficient ($1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$).

Assay of diene conjugates. The method of Recknagel and Ghoshal [8] was used to measure diene conjugate content in membrane lipids. Lipids were extracted from the yeast cells using the methodology of Folch et al. [9] under nitrogen stream. The absorbance was noted at 230 nm against cyclohexane as a blank. The content of diene conjugates was calculated using the molar extinction coefficient of $2.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Total proteins were extracted using Schmidt-Thannhauser method as modified by Munro and Fleck [10] and were estimated by the method of Lowry et al. [11].

Extraction and analysis of total lipids. Lipids were extracted according to the method of Folch et al. [9] and analyzed. The estimation of phospholipid was based on their phosphorus content measured by Ames' method [12].

Individual phospholipids were separated by TLC on silica gel-G coated plates containing 1% Na_2CO_3 using $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$ (65:35:5 v/v) solvent system. The spots of various phospholipids were detected by iodine vapours and identified by comparing their Rf values with that of standard and confirmed by various spray reagents as described by Mangold [13]. Total and esterified sterols were estimated using the method of Sperry and Webb [14]. Free sterols were precipitated by digitonin (1%) before the estimation of esterified sterols. Glycolipids were estimated as described by Dubois et al. [15]. The method of Gaunt and Barlow [16] was followed for the estimation of α -tocopherol.

Assay of catalase (EC 1.11.1.6). Homogenate of the yeast cells was prepared in 0.1 M phosphate buffer, pH 6.8, and centrifuged at 7000 g for 20 min. The clear supernatant was used for the assay of catalase [17].

The reaction mixture contained 1 ml of 2 mM hydrogen peroxide, 3 ml of phosphate buffer (0.1 M, pH 6.8) and 1 ml of enzyme preparation (0.1–0.7 mg protein/ml). The reaction mixture without substrate was pre-incubated at 25°C for 5 min. The reaction was started by adding 1 ml of hydrogen peroxide. After incubation at 25°C for 10 min, the reaction was stopped by adding 5 ml H_2SO_4 (1.5 N). Residual hydrogen peroxide was estimated by titrating against 0.5 N KMnO_4 . In case of control, the

reaction was stopped at "zero" time. Standard curve for hydrogen peroxide was prepared by taking different concentrations of hydrogen peroxide and titrating against 0.5 N KMnO_4 . The enzyme activity is expressed as μmoles of hydrogen peroxide degraded/mg protein/min.

Results and discussion

Lipid peroxidation of *S. cerevisiae* as indexed from MDA as well as diene conjugates showed a marked increase in ethanol-treated cells and the increase was concentration-dependent. Yeast cells treated with 12% ethanol had an MDA level about 10 times that of the control when the results were represented on per million cells basis (Table I). Alcohol-treated animal cells also exhibited higher levels of lipid peroxidation [18, 19]. The increased lipid peroxidation might be the result of enhanced free radical production in ethanol treated cells, as Davis et al. [20] had reported that ethanol induced the generation of reactive oxygen intermediates such as superoxide radical, hydrogen peroxide and hydroxyl anion. All these species are important initiators of lipid peroxidation. Secondly, the compositional and structural changes in membranes of ethanol treated cells might be responsible for their increased peroxidation.

Table I

Malondialdehyde (MDA) content of yeast cells treated with ethanol

Alcohol concentration (% v/v)	MDA (nmoles)			Diene conjugates (nmoles/100 mg dw)
	Per 100 mg dw	Per million cells	Per mg phospholipids	
0	5.02 $\pm$ 0.09	1.21 $\pm$ 0.03	2.50 $\pm$ 0.09	121.20 $\pm$ 4.41
4	6.21 $\pm$ 0.09***	2.18 $\pm$ 0.10***	4.73 $\pm$ 0.21***	148.76 $\pm$ 3.81**
8	6.66 $\pm$ 0.10***	3.92 $\pm$ 0.08***	11.92 $\pm$ 0.23***	168.67 $\pm$ 4.21***
12	6.96 $\pm$ 0.12***	11.58 $\pm$ 0.33***	29.24 $\pm$ 0.27***	186.42 $\pm$ 5.89***

Results are mean $\pm$ SD of four experiments.

The values are significantly different from the control at ** $p < 0.005$; *** $p < 0.001$

α -Tocopherol, a potent lipid soluble antioxidant, is essentially the sole protective agent present within the membranes [21]. The amount of α -tocopherol was 0.47 $\mu\text{g/ml}$ of total lipids in untreated cells (Table II). Tocopherol content showed a significant decrease in the lipids of cells treated with ethanol. In addition, α -tocopherol affects the stability of membranes [22, 23].

Table II

Variations in lipid composition of S. cerevisiae with ethanol treatment

Alcohol	Total lipids	Phospholipids		Total sterols	Esterified sterols	Free sterols	Glycolipids	α -tocopherol	Free sterol :	Phospholipid :	Glycolipid :
concentration									Phospholipid	Protein ratio	Phospholipid
% v/v	% dry weight	% dry weight	% total lipids	% total lipids	% total lipids	% total lipids	% total lipids	mg/g total lipids	molar ratio		ratio
0	4.23	1.95	49.44	15.32	7.03	8.29	18.97	0.47	0.33	0.061	0.38
	± 0.09	± 0.07	± 1.91	± 0.61	± 0.32	± 0.33	± 0.83	± 0.03			
4	2.53	1.33	46.04	14.90	6.95	7.95	27.58	0.35	0.34	0.046	0.60
	$\pm 0.08^{***}$	$\pm 0.05^{***}$	$\pm 1.81^*$	± 0.53	± 0.30	± 0.24	$\pm 0.99^{***}$	$\pm 0.02^{***}$			
8	1.67	0.56	32.42	12.42	5.27	7.15	32.62	0.27	0.43	0.019	1.01
	$\pm 0.07^{***}$	$\pm 0.06^{***}$	$\pm 1.51^{***}$	$\pm 0.35^{**}$	$\pm 0.31^{**}$	$\pm 0.25^*$	$\pm 1.03^{***}$	$\pm 0.02^{***}$			
12	1.33	0.32	23.14	10.78	4.45	6.33	24.07	0.21	0.53	0.007	1.04
	$\pm 0.07^{***}$	$\pm 0.05^{***}$	$\pm 0.85^{***}$	$\pm 0.49^{***}$	$\pm 0.23^{***}$	$\pm 0.42^{**}$	$\pm 0.87^{**}$	$\pm 0.01^{***}$			

Results are mean $\pm$ SD of four experiments.The values are significantly different from the control at $^*p < 0.01$; $^{**}p < 0.005$; $^{***}p < 0.001$

Hydrogen peroxide plays an important role in lipid peroxidation particularly in free radical generation [24]. Therefore, the role of catalase which detoxifies hydrogen peroxide [25] was studied in alcohol-induced lipid peroxidation. Catalase activity showed an inverse relationship with the alcohol concentration (Fig. 1). The decrease in catalase activity might be due to alcohol inactivation on this enzyme.

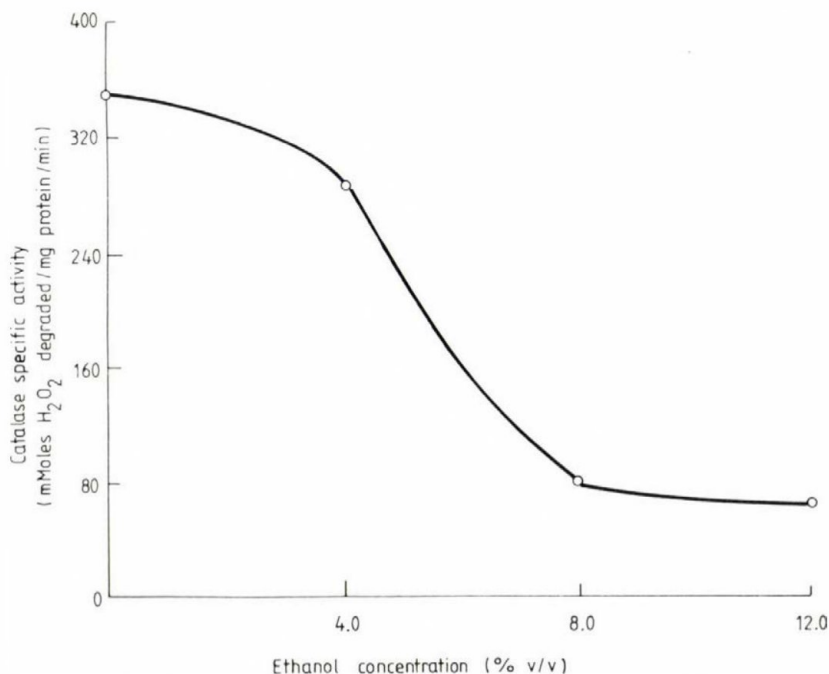


Fig. 1. Effect of ethanol concentration on specific activity of catalase

Ethanol brought some changes in lipid composition of *S. cerevisiae* (Table II). Phospholipids decreased in alcohol-treated yeast cells. Nandini-Kishore et al. [26] have reported that alcohol(s) inhibit phospholipid biosynthesis. Total sterols also decreased in ethanol-treated cells. Sterol:phospholipid ratio decreased at 4%, however it increased at 12% ethanol.

It may be an adaptive measure to decrease fluidity of the membranes. Phospholipid:protein ratio decreased with increase in alcohol concentration. The results are similar to that of Galeotti et al. [27], who showed that a decrease in this ratio was accompanied with an increase in MDA content. Glycolipid content of *S. cerevisiae* cells increased from 18.97 ± 0.83 to $32.62 \pm 1.03\%$ of total lipids when treated with 8% (v/v) ethanol but a further rise in ethanol concentration to 12% (v/v) decreased

glycolipids to $24.07 \pm 0.87\%$. However, glycolipid:phospholipid ratio increased with an increase in alcohol concentration. Glycolipids due to higher transition temperature have stabilizing effect in the membrane [28]. This relative increase in glycolipids might be an adaptive mechanism for the yeast to counter the membrane destabilizing effect of ethanol.

Table III

Effect of alcohol intoxication on phospholipid composition of S. cerevisiae

Alcohol concentration (% v/v)	Relative percentage						PC:PE ratio
	DPG	PE	PC	PS	PI	LPL	
0	3.56	13.02	67.36	5.47	7.39	3.06	5.17
4	7.20	12.47	51.57	10.19	11.09	7.49	4.14
8	16.28	14.17	39.78	14.28	6.66	8.09	2.81
12	20.09	19.48	35.81	6.56	8.95	9.11	1.83

Results are mean of four experiments.

DPG: Diphosphatidylglycerol; PE: Phosphatidylethanolamine; PC: Phosphatidylcholine;
PI: Phosphatidylinositol; PS: Phosphatidylserine; LPL: Lysophospholipids

Relative amount of individual phospholipids also showed variations (Table III) in the yeast cells treated with alcohol. Diphosphatidylglycerol content increased significantly with increase in alcohol concentration. Ethanol has been shown to disorder biological membranes [4]. The increased amount of diphosphatidylglycerol might be conferring tolerance to ethanol because of their stabilizing effect on membranes as proposed by Ellingson et al. [29]. Phosphatidylethanolamine level increased at 12% ethanol. The amount of phosphatidylcholine decreased on increasing the concentration of ethanol. Overall, the ratio of phosphatidylcholine:phosphatidylethanolamine decreased. It is well known that low amount of phosphatidylcholine decreases the membrane fluidity [30]. Therefore, this trend may also be an adaptation by membranes to counter increased fluidizing effect of alcohol. The amount of phosphatidylserine and phosphatidylinositol did not show a definite pattern. However, lysophospholipids increased with alcohol concentration. This increase may be due to an increased activity of lipolytic enzymes as reported by Alling et al. [31].

The unsaturation of fatty acids was enhanced in yeast cells under alcohol stress (Table IV). Unsaturated fatty acyl residues are more susceptible to peroxidation and secondly *cis* double bonds disturb the ordered packing of fatty acyl residues in lipid bilayer and increase membrane lipid fluidity. Both these effects may be contributing to an increase in peroxidation level in ethanol-treated cells.

Table IV

Variations in fatty acid composition of S. cerevisiae cells with ethanol treatment

Ethanol concent- ration (% v/v)	Fatty acids (Relative percentage)													Unsat- uration index
	12:0	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	22:0	22:1	22:2	24:1	
0	4.87	6.99	1.58	26.18	23.36	3.67	14.60	6.18	4.25	3.17	0.20	1.81	3.01	71
4	3.30	5.01	1.20	23.00	26.12	2.21	16.19	8.61	7.33	2.05	0.25	1.61	3.11	89
8	2.71	2.75	1.20	21.01	27.80	0.91	17.10	11.19	10.21	1.21	0.21	1.31	2.21	104
12	2.11	1.90	1.41	20.01	28.12	0.70	17.79	12.11	11.91	0.41	0.30	1.20	2.01	112

It can be concluded that ethanol damages membranes by inducing lipid peroxidation. Antioxidant mechanisms (catalase activity and tocopherol(s) content) of yeast cells fail to counter the effect of ethanol in the present findings.

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AUTOLOGOUS ACTIVATED LYMPHOCYTE THERAPY IN A COMMUNITY HOSPITAL*

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In the authors' institution patients suffering from metastatic melanoma, renal cell carcinoma or mesothelioma, resistant to conventional therapeutic modalities, are treated with adoptive immunotherapy. Tumour infiltrating lymphocytes (TIL) are prepared from surgical samples and expanded *ex vivo* in the presence of recombinant interleukin-2 (rIL-2). When sufficient amount of cells are available (5×10^9 – 10^{10}) they are being reinfused. The patients also receive rIL-2 subcutaneously to support the activity and proliferation of reinfused TIL, and to avoid side effects caused by bolus or continuous intravenous administration. Leukapheresed lymphocytes activated by conditioned medium from OKT3 stimulated autologous lymphocytes and rIL-2 (autologous activated lymphocytes, AAL) are used as an alternative when TIL is not available or until it can be produced in sufficient amount. Subcutaneous IL-2 and oral cimetidine are also administered to support the reinfused AAL and to inhibit activation of CD8⁺ suppressor cells, respectively. Expression of activation markers CD25 and HLA-DR are monitored by flow cytometry as well as cytotoxicity is measured against K562 (NK specific target), HeLa (AALT specific) and against allogeneic or autologous tumour cell targets with a non-radioactive test. Methods are discussed by which the therapeutic efficiency of infused lymphocyte preparations can be improved.

The discoveries of the sixties and seventies (different lymphocyte subsets, lymphokines, monoclonal antibodies, etc.) reinforced the vision of adoptive immunotherapy of malignant diseases as a prospective therapeutic modality with high potentials. However, adoptive immunotherapy became a reality only after recombinant cytokines became available and licensed for human therapeutic use. Interleukin-2 (available as a recombinant product, "Proleukin for injection", Chiron Corp.), expands NK cells and T lymphocytes very efficiently *in vitro* and *in vivo*. In adoptive tumour immunotherapy the *ex vivo* IL-2 activated and expanded peripheral blood lymphocytes (a heterogeneous lymphocyte population) or a more or less tumour-specific lymphocyte population are being reinfused into patients with metastatic disease (for review see 1, 2).

*This paper was written in honour of the memory of Professor Zoltán Alföldy (1902–1992) director emeritus of the Institute of Microbiology Semmelweis University Medical School in Budapest (Hungary)

TIL are prepared from surgically removed tumour samples [3]. The mononuclear cell population is *in vitro* expanded in the presence of IL-2 with the final goal of reinfusion into the patient. After a few weeks with IL-2 the culture contains no significant amount of NK cells but only T-lymphocytes. The TIL population is believed to consist of tumour-specific T lymphocytes, in particular MHC-restricted CD8⁺ cytotoxic T cells (CTL) which mediate autologous tumour cell lysis [4–6]. This tumour-specificity explains its higher (50–100-fold) efficiency over the non-tumour specific lymphokine activated killer (LAK) lymphocytes [7]. The use of TIL is not restricted to therapeutic purposes as radioisotope labelled tumour-specific lymphocytes are used to visualize metastases [8]. TIL transduced with a defective retrovirus vector carrying the neo^R gene can be detected in the circulation, showing that the reinfused cells can proliferate for extended periods of time [9].

Lymphocytes obtained from lymph nodes draining the tumour site also represent a population of tumour-specific immune cells: B cells may be used to construct hybridomas and T cell specificity can be increased by "priming" with killed autologous tumour cells or cell extracts [10, 11].

LAK cells are obtained from the peripheral blood by leukapheresis. These cells are activated to proliferate *in vitro* by different ways. Treatment with cytokines (most frequently IL-2) or with the pan-T cell specific monoclonal antibody OKT3 results in a cell-population with broad lytic activity against tumour cells [12–14]. Among LAK cells it is the activated NK cell which is mostly responsible for the killing effect (for review see 2).

In the authors' institution patients suffering from metastatic malignant melanoma and renal cell carcinoma participate in the adoptive immunotherapy clinical trials.

Materials and methods

Patients. Surgically removed tumours of patient with malignant melanoma, renal cell carcinoma or bronchiolo-alveolar carcinoma are collected for TIL preparation. Patients with metastatic disease, who failed standard treatment, are selected to participate in the adoptive tumour immunotherapy study. The protocol is approved by the institutional reviewing board and written informed consent is obtained from all patients before entering the program.

TIL production. TIL cultures are prepared from surgically removed tumours in the following fashion. Mechanically disrupted tissue pieces are placed in T-75 tissue culture flasks in TIL medium, consisting of AIM-V medium (GIBCO, specifically designed to support LAK and TIL cultures) or RPMI-1640 supplemented with 10% fetal calf serum, LAK cell conditioned medium (20% of final volume) and 1–4000 U/ml of IL-2 and the cultures are kept in 5% CO₂ atmosphere at 37 °C. Lymphocytes, migrating out of the tumour tissue are collected separated from cell debris and dead cells by Ficoll–Paque (Pharmacia) density gradient centrifugation. Washed lymphocytes are transferred to 24 well tissue culture microplates in 0.5×10⁶/ml density and fed twice weekly maintaining that concentration with fresh TIL medium. Non-viable cells are removed by Ficoll–Paque centrifugation when necessary. When sufficient amount (usually between 10⁹–10¹⁰ cells) is produced the cells are collected and cryopreserved until further use.

The cells in culture are collected four days before reinfusion, washed vigorously in Hanks' solution and cultured in Fenwal polyolefine gas permeable tissue culture bags in TIL medium containing autologous or pooled human AB serum, previously tested for the absence of blood-borne pathogens (South West Florida Blood Bank), 20% conditioned medium (also prepared with human AB

serum) and IL-2. For reinfusion the cells are washed thrice and resuspended in Ringer-lactate with 0.5% human serum albumin and transported to the infusion room.

Production of autologous activated lymphocytes. Patients, from whom there is no surgical sample available, receive autologous activated lymphocytes (AAL). The method published by Osband and colleagues is used for AAL production with some modifications [13, 15]. Buffy coat mononuclear cells are obtained by leukapheresis from the patients' peripheral blood. Cells from the first leukapheresis are used to produce conditioned medium for AAL production. The yield of mononuclear cells after 5 days in culture in the presence of the 2–3% granulocyte and about 10% red blood cell contamination is superior as compared to Ficoll–Paque separated mononuclear cell populations [14, 16]. Therefore the buffy coat is allowed to sediment and the supernatant containing most of the mononuclear cells, some granulocytes and red blood cells is saved. The red blood cell sediment is diluted with two volumes of Hanks' solution and is centrifuged on Ficoll–Paque gradient to recover the trapped mononuclears. Cells collected from the gradient interface are combined with the first cell population and washed in low speed to remove platelets with the supernatant. The cells are suspended ($2 \times 10^6/\text{ml}$) in AIM-V medium containing 2% of patient's heat-inactivated serum and OKT3 mononuclear antibody (10 ng/ml, Ortho Diagnostics) is added. The cells are transferred to Fenwal tissue culture bags and cultured at 37 °C. After 3 days the cultures are centrifuged, the supernatant is filtered through a 0.22 μm cellulose acetate filter, aliquoted and kept frozen at –20 °C until its further use as conditioned medium.

Further buffy coats are collected for AAL production in 2 weeks intervals. The mononuclear cells are prepared as above and resuspended in AIM-V medium containing 20% autologous conditioned medium, 2% autologous serum, 4000 U/ml IL-2 and 5×10^{-5} mol/l cimetidine at 3×10^6 cell/ml density and cultured on 37 °C.

On the fourth day an aliquot is removed for microbiological sterility test. The culture is terminated on the 5th day, the cells are carefully collected and washed, resuspended in 50–100 ml of Ringer-lactate solution with 5% human serum albumin and reinfused.

Toxicity and side effects, medication. Virtually no toxicity related to the reinfusion of the activated lymphocytes could be identified. Fever, chills, skin rash, anxiety and dizziness are related to the subcutaneous IL-2 treatment. These reactions are reversible and can be treated or prevented by medications.

The patients receiving TIL or LAK therapy are taking oral cimetidine (600 mg q6 h) to reduce suppressor T-cell activity and are also receiving subcutaneous rIL-2 treatment (5 million U/day) on the day of reinfusion and the following 10 days. Patients' response is monitored by imaging measurements of the change in size of the tumour or metastasis.

Target cells and measurement of cytotoxicity. Lymphoblastoid cell line K562 was kind gift of P. Medveczky (Department of Microbiology, University of South Florida College of Medicine), and maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS, from UBI), glutamine and antibiotics. HeLa cells were cultured in Dulbecco's modified minimal essential medium (DMEM). Whenever is possible, autologous melanoma cell lines are also used as target cells. These cell lines are established in our laboratory, using RPMI-1640 medium supplemented with 15% FCS, non-essential amino acids, sodium pyruvate and antibiotics [17]. Overgrowing fibroblasts are inhibited with 100 $\mu\text{g}/\text{ml}$ Geneticin (GIBCO) applied for 2 days [18]. The presence of the S-100 protein and reactivity with the HmB45 monoclonal antibody was tested by immunocytochemistry and proved the melanocytic origin of the obtained cell line.

Cytotoxicity against K562 (NK target), autologous and/or allogeneic tumour cell lines, HeLa or Raji cells is estimated in a non-radioactive test according to the manufacturer's recommendations with some modifications (Cyto Tox 96™ Non-Radioactive Cytotoxicity Assay, Promega). The test is based on measurement of the activity of the cytosolic enzyme, LDH, released from killed target cells [19–21]. As the background was high due to the phenol red in the culture medium and LDH activity in FCS we use RPMI-1640 without phenol red, complemented with 1% bovine serum albumin and glutamine.

Flow cytometry. To test the proportion of different lymphocyte subsets and the presence of activation markers flow cytometry assay is performed using a combination of monoclonal antibodies

specific to CD3 (pan-T); CD4 (T-helper); CD8 (T-suppressor/cytotoxic); CD16+56 (NK); CD20 (B-cell) as well as to CD25 (IL-2 receptor) and HLA-DR as activation markers (Becton-Dickinson). Recently another activation marker, CD38 is tested as well. The test is read on FACSCAN using the software Lysis II, both from Becton-Dickinson.

Results and discussion

Peripheral blood mononuclear cells, collected by leukapheresis are prepared for AALT for patients suffering of metastatic diseases. The number of cells collected by leukapheresis for AALT is between 5×10^8 and 3×10^9 . A 1.5–2.2-fold increase in cell number is regularly achieved during the 5 days culture period.

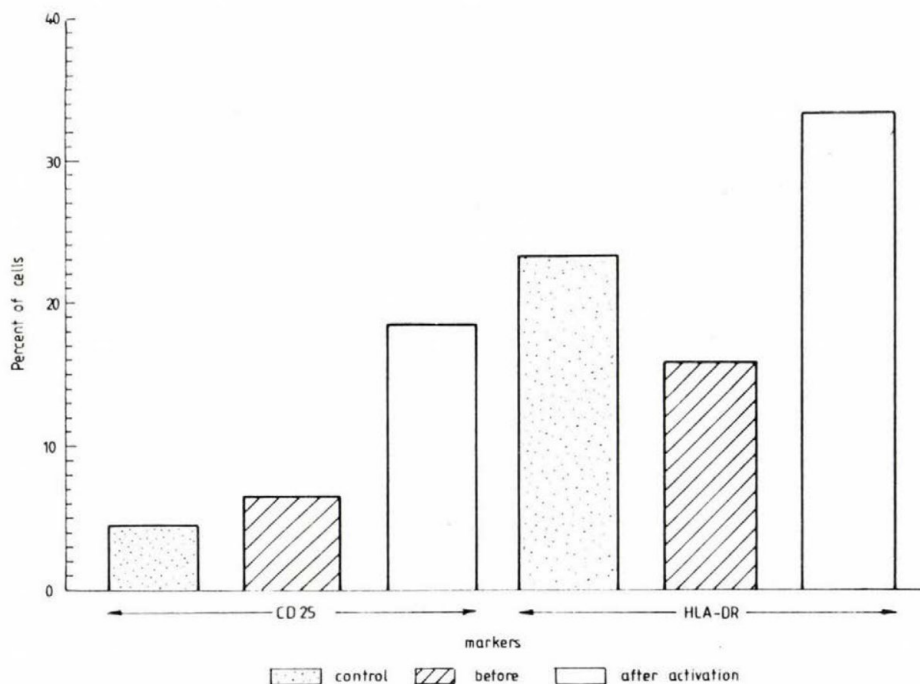


Fig. 1. Expression of IL-2 receptor and the HLA-DR activation antigen on lymphocytes of patients with malignant diseases, before and after activation with autologous conditioned medium and IL-2, as well as on lymphocytes of controls. The results are expressed in percentage of cells staining positively with the appropriate antibodies by flow cytometry

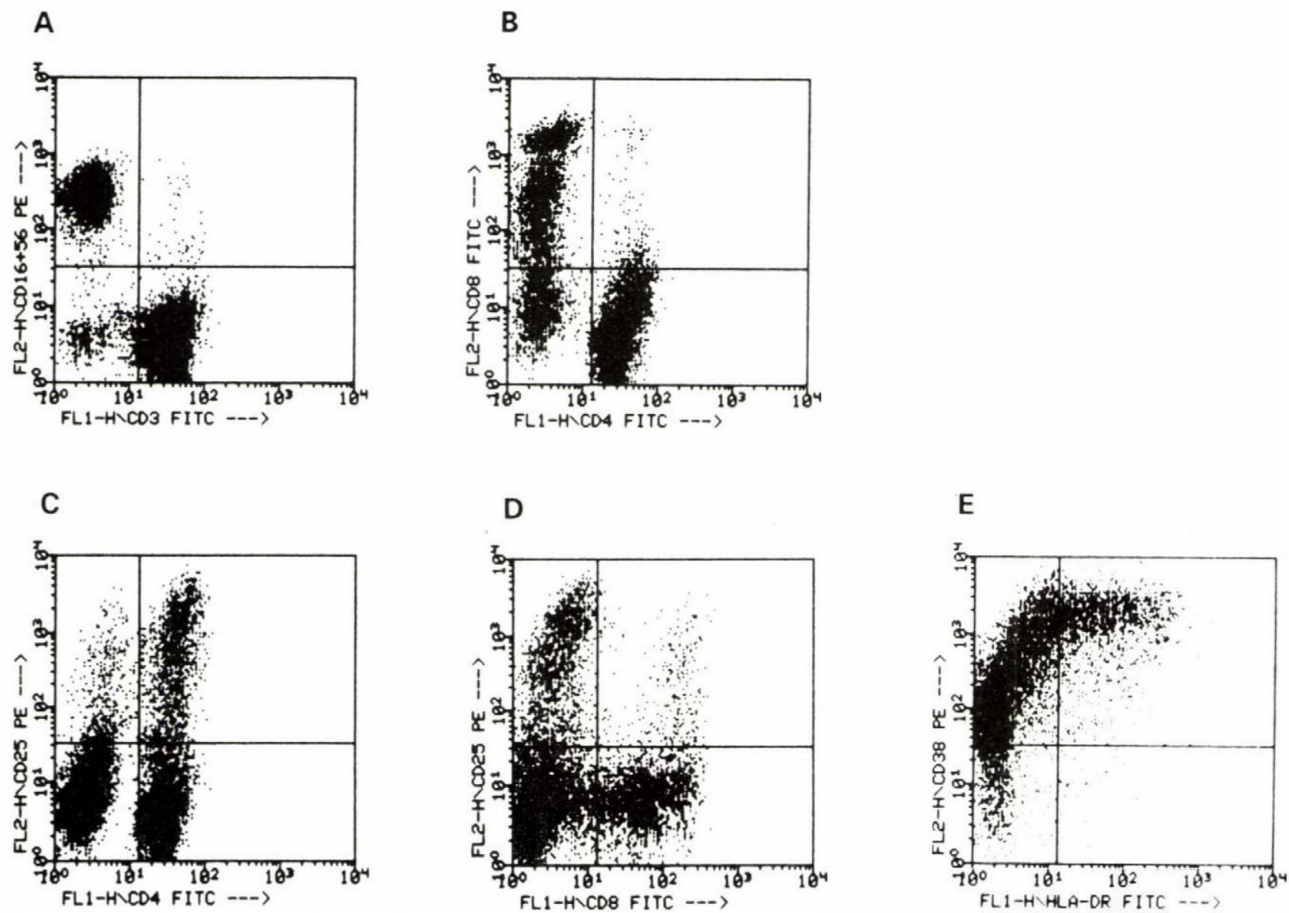


Fig. 2. Cytotoxicity of lymphocytes of a patient suffering from malignant melanoma, before and after ex vivo activation, against K562, HeLa and autologous tumour cell targets

The phenotype of reinfused activated lymphocytes is studied by flow cytometry. While the number of CD4 and CD8 marker-expressing cells do not change significantly during the incubation period, the proportion of cells expressing activation marker CD25 (IL-2 receptor) is increasing from 6.4% to 18.7%. The proportion of HLA-DR expressing cells is doubled to 33.7%. Compared to normal controls (10 persons), CD25 expression is moderately higher (probably due to the IL-2 treatment) and HLA-DR expression is lower in the collected buffy coat mononuclear cells of cancer patients (Fig. 1). Typical result of two-stain flow cytometry of activated lymphocytes of a patient suffering from renal cell carcinoma is shown on Fig. 2. NK cells represented 27% of the reinfused cells (Fig. 2A), 51% was CD4⁺ and 30% CD8⁺ cell (Fig. 2B). The IL-2 receptor was expressed on 16% of CD4⁺ and 2.8% of CD8⁺ lymphocytes (Fig. 2C and D, upper right field). The CD38 activation marker was expressed on 83% of all cells and 17% expressed HLA-DR as well (Fig. 2E upper fields and right field, respectively).

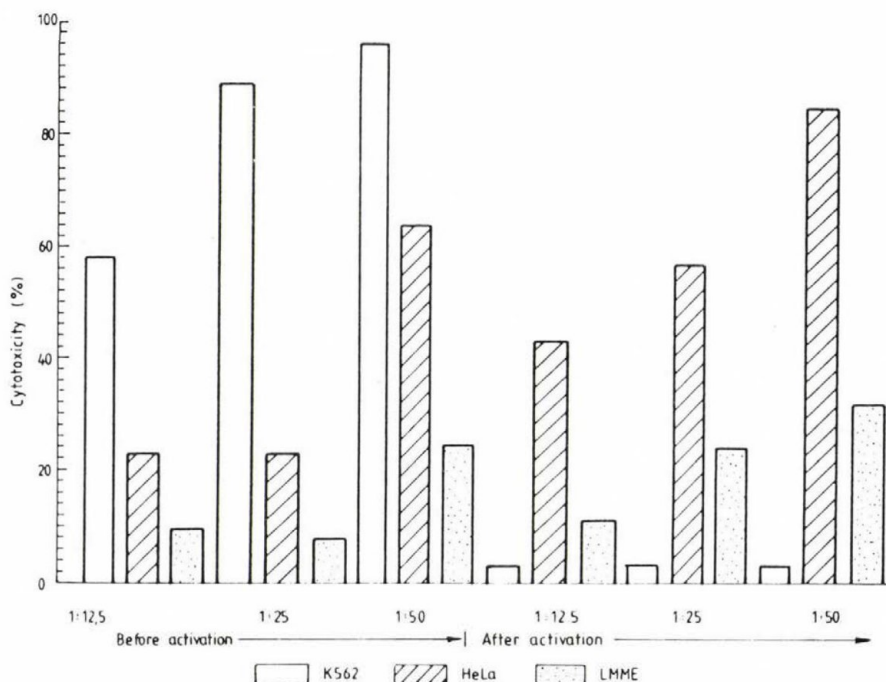


Fig. 3. Two-stain flow cytometric analysis of activated lymphocytes of a patient suffering from renal cell carcinoma metastatic to the lungs. (See explanation in the text)

Cytotoxicity against K562 (NK-specific) and HeLa (LAK-specific) target cells is measured in a non-radioactive assay. As cytotoxicity against K562 is decreasing, HeLa cells are killed in higher percentage by activated then by peripheral blood

mononuclears. Cytotoxicity against autologous tumour cells is greatly improved at target:effector ratio of 1:12.5 and 1:25, moderately at 1: 50 (Fig. 3).

Establishment of TIL cultures is attempted from all surgically removed tumour samples. When sufficient amount of cells is produced, samples are cryopreserved for later initiation of TIL cultures. When the cell number in cultures reaches 10^{10} , they are being reinfused.

Flow cytometry analysis of several TIL cultures revealed that they are $CD4^+$ T-helper cells, not the primary cytotoxic lymphocyte population. These cells are, however, capable of attacking and destroying tumour cells as seen on Fig. 4. The anti-tumour activity was tested in vivo as well, TIL were reinjected into the peritoneal cavity of the patient suffering from malignant melanoma involving the peritoneum. Ascites drawn 24 h later showed the presence of lymphocytes attacking tumour cells (Fig. 5). Reinfusion of $CD4^+$ TIL (3.5×10^9 cells) to a patient suffering from progressing metastatic renal cell carcinoma stabilized the disease. Others also reported the effectiveness of $CD4^+$ lymphocytes to generate clinical response [22].

Experimental results in mice showed that neither $CD4^+$ nor $CD8^+$ lymphocytes could produce tumour lysis without the other. Although it is the $CD8^+$ cytotoxic lymphocyte which is most responsible for tumour lysis, the role of $CD4^+$ lymphocytes is more than mere IL-2 production. Addition of IL-2 can not substitute for the physical presence and cellular interaction of $CD4^+$ lymphocytes with their $CD8^+$ partner [23].

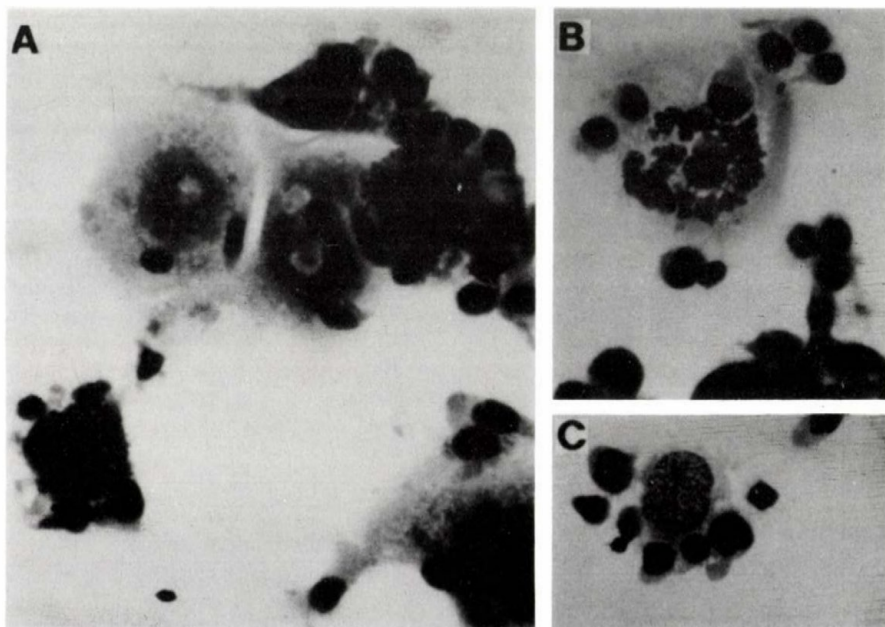


Fig. 4. TIL of a patient with metastatic melanoma attacking autologous tumour cells in culture. (Giemsa staining of tissue culture chamber slide)

There is virtually no side effect as a direct consequence of lymphocyte reinfusion. If toxicity experienced, it is mostly due to the administration of IL-2 and presents itself as chills, fever, local erythema, anxiety and dizziness; these are reversible and can be treated or prevented by medication.

Difficulties to raise sufficient amount of TIL may arise when adequate size of tumour sample is not available or the sample contains necrotic tissue. About 20% of the cultures die without yielding sufficient number of cells for therapy. Collection of sufficient number of PBMC by leukapheresis might be hampered by haematologic consequences of earlier chemotherapy or radiotherapy.

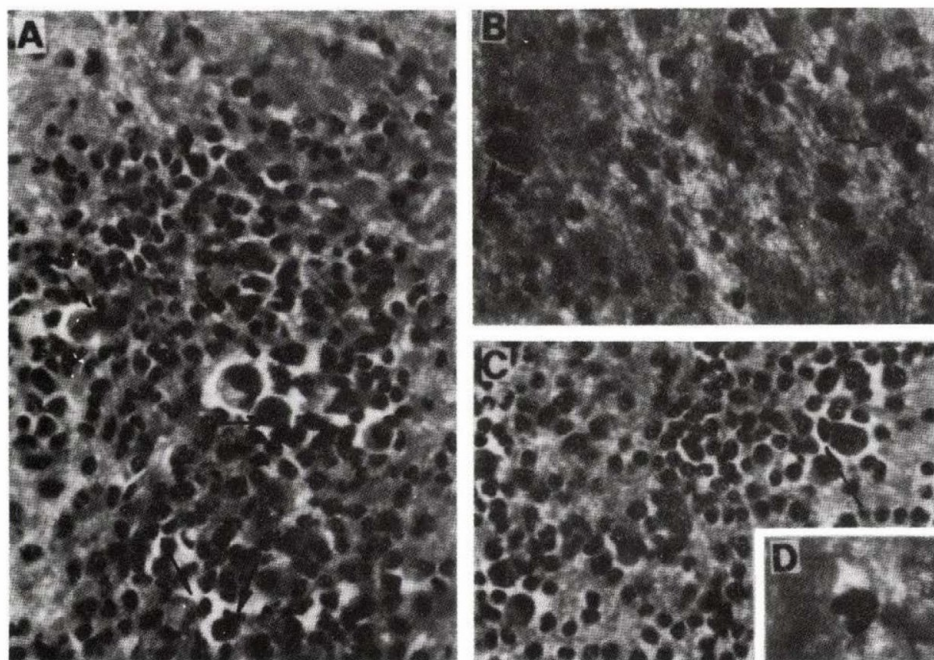


Fig. 5. TIL were injected to the peritoneal cavity of a patient with melanoma metastatic to the peritoneal cavity. Sample drawn 24 h later shows the presence of lymphocytes attacking tumour cells (arrows). (Paraffin section, hematoxylin-eosin staining)

The moderate effect of LAK and TIL therapy (20–25% combined complete and partial response, published by several centres) on patients with metastatic malignant melanoma and renal cell carcinoma is somewhat disappointing [22, 24, 25]. There are ways, however, which might promise improvement.

(i) Although it is accepted that LAK lymphocytes and specially TIL are targeted to the site of the tumour through circulation, direct delivery of these cells to the tumour

site (i.e. administration through the artery supplying the tumour) might improve efficiency [8, 26–28].

(ii) Use of autologous tumour cell “primed” LAK lymphocytes, whenever it is possible, increases the specificity of killing [29, 30]. Cloned tumour-specific T-lymphocytes execute tumour killing more efficiently than a heterogeneous lymphocyte population.

(iii) Several lymphokines are known to synergize with IL-2 to activate NK and T lymphocytes. Good candidates are IL-6, IL-10, IL-12, interferon-alpha and interferon-gamma as well as TNF-alpha and TNF-beta [31–33]. This effect can be taken advantage of during activation and administration of LAK and TIL.

(iv) TIL can be genetically modified by transducing them with retroviral vectors carrying the IL-2 or TNF genes, making them self-supportive and highly cytotoxic [9, 34].

These alternatives are being considered to improve the efficiency of adoptive immunotherapy.

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IS *ESCHERICHIA COLI* ATCC 25922 A COLICIN PRODUCING STRAIN ? (A NOTE)

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(Received June 17, 1993)

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The well-characterized strain of *Escherichia coli* ATCC 25922 recommended and widely used as a control Gram-negative bacterium for various laboratory experiments, especially for antibiotic susceptibility assays, was found to be antagonistic against another *E. coli* strain which is uniquely susceptible to fusidic acid, an antibiotic mainly active against Gram-positive bacteria. This antagonistic property appears to be selective, since it was not found against other *E. coli* or *Staphylococcus aureus* strains.

While performing experiments aimed at testing an unknown mold strain for antibiotic production against various *Escherichia coli* strains, an unexpected observation was made. On the agar surface the *E. coli* ATCC 25922 strain apparently suppressed the growth of the fusidic acid-susceptible *E. coli* strain which was streaked radially to the mold colony, accidentally close to each other. This chance observation prompted designed experiments for further study of this interesting phenomenon. This note reports the initial results.

Materials and methods

Bacterial strains. *E. coli* ATCC 25922 was purchased from American Type Culture Collection [1]. The *E. coli* which was made fusidic acid susceptible by mutagenesis after treatment with the potent mutagenic agent, nitrosoguanidine [2] was provided by Dr. Julian Davies [3]. Fusidic acid is known to be active only against Gram-positive bacteria [4]. Other *E. coli* strains, including the strong beta-lactamase-producing No. 211 strain and the *Staphylococcus aureus* strains used were those described in the literature [5–7]. For the study of the antagonistic activity of *E. coli* ATCC 25922, the various forms of the conventional agar-diffusion methods were used. These are shown in Figs 1 and 2. The studies were performed repeatedly on the usual commercial media, such as Mueller–Hinton agar, nutrient agar, trypticase–soy agar as well as on the semisynthetic peptone–glucose agar [5].

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Results and discussion

The inhibitory (antagonistic) effect of *E. coli* ATCC 25922 against the fusidic acid-susceptible *E. coli* strain is demonstrated in Figs 1 and 2. Figure 1 shows that a streak of *E. coli* ATCC 25922, inoculated on Mueller-Hinton agar and grown overnight at 37 °C, inhibits the growth of several dilutions of the fusidic acid-susceptible *E. coli* strain streaked perpendicularly and incubated overnight at 37 °C. There is no growth in the immediate vicinity of the *E. coli* ATCC 25922 streak and in a certain distance, as far as the inhibitory substance diffuses in the agar.



Fig. 1. *E. coli* ATCC 25922 grown on Mueller-Hinton agar inhibits the growth of the fusidic acid susceptible *E. coli* strain (many dilutions). There is no growth close to and at a certain distance from the colony of *E. coli* ATCC 25922. Then as the concentration of the diffusible inhibitory substance (colicin) concentration diminishes, some growth starts. Full growth is seen at a further distance

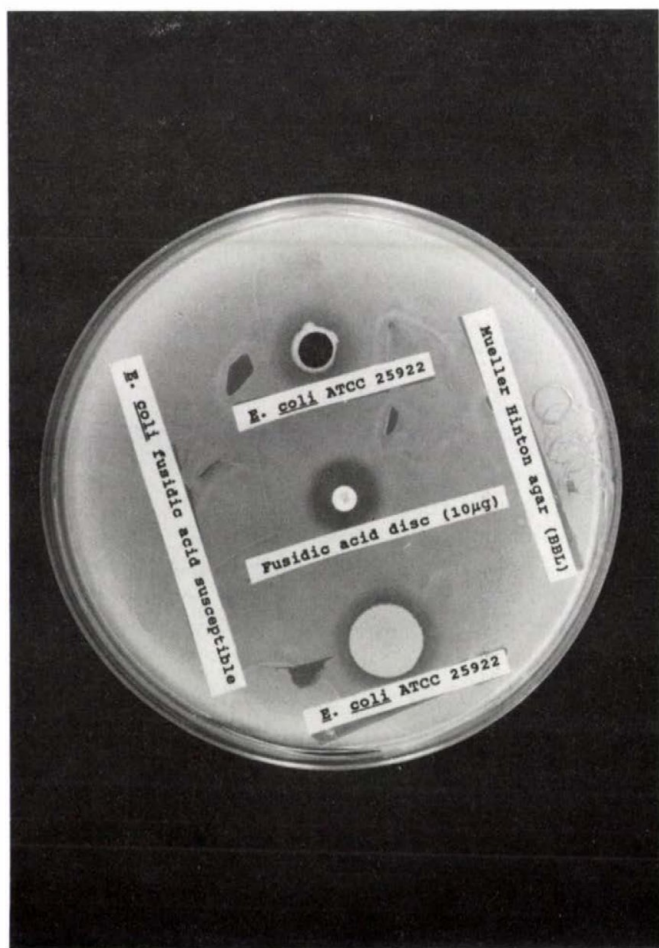


Fig. 2. Inhibition zones caused by *E. coli* ATCC 25922 when placed either in the hole (upper) or directly onto the surface (lower) of the inoculated and evenly grown fusidic acid susceptible *E. coli*. In the middle, the inhibition zone is produced by a fusidic acid susceptibility disk containing 10 µg

Figure 2 demonstrates, by using another form of the agar diffusion method, that *E. coli* ATCC 25922 inhibits the growth of the fusidic acid-susceptible *E. coli* strain. The fusidic acid-susceptible *E. coli* was applied as a lawn onto the Mueller-Hinton agar plate. After drying the surface, *E. coli* ATCC 25922 suspension was immediately applied to the lawn in two ways: in a hole produced by a cork-borer (upper part) and also directly onto the dry surface (lower part).

After overnight incubation at 37 °C, there were well-defined inhibition zones at both places, demonstrating the production by *E. coli* ATCC 25922 of a diffusible inhibitory substance. In the middle of the plate, the inhibitory zone produced by a fusidic acid-containing (10 µg) susceptibility disk demonstrates that the *E. coli* strain retains its susceptibility to fusidic acid induced by mutagenesis even after numerous transfers over the years. This newly acquired genetic property appears to be stable.

The inhibitory substance produced by *E. coli* ATCC observed and described for the first time in this paper may be a non-mammalian or non-insect defensin-like molecule [8–10], or most probably a colicin. Colicins (belonging to the group of bacteriocins) are antibiotic-like soluble substances produced by certain *E. coli* strains, having inhibitory activity against some other strains of the same or related species [11–13].

The antibacterial activity of *E. coli* ATCC 25922 was found to be very specific. It appears to inhibit only the fusidic acid-susceptible mutant *E. coli* strain. It showed no inhibition, in similar testing format as shown in Fig. 1, against other *E. coli* and *S. aureus* strains tested so far.

The fact that *E. coli* ATCC 25922 is resistant to its own inhibitory substance, can be used as an argument that the product is a colicin [14, 15]. In this case, since more than 20 colicin subtypes are reported, a detailed study of the inhibitory substance (colicin) produced by *E. coli* ATCC 25922 is required for its exact characterization [16]. Until this work is done, it seems to be important to draw the attention of those investigators who use *E. coli* ATCC 25922 for antibiotic susceptibility assays and other microbiologic studies to consider the recently recognized property of the strain, i.e., that it produces an antimicrobial substance, and probably colicin.

Acknowledgement. The author wishes to express his thanks to Dr. Julian Davies, Institute Pasteur, Paris, France for kindly providing a subculture of his fusidic acid-susceptible mutant *E. coli* strain.

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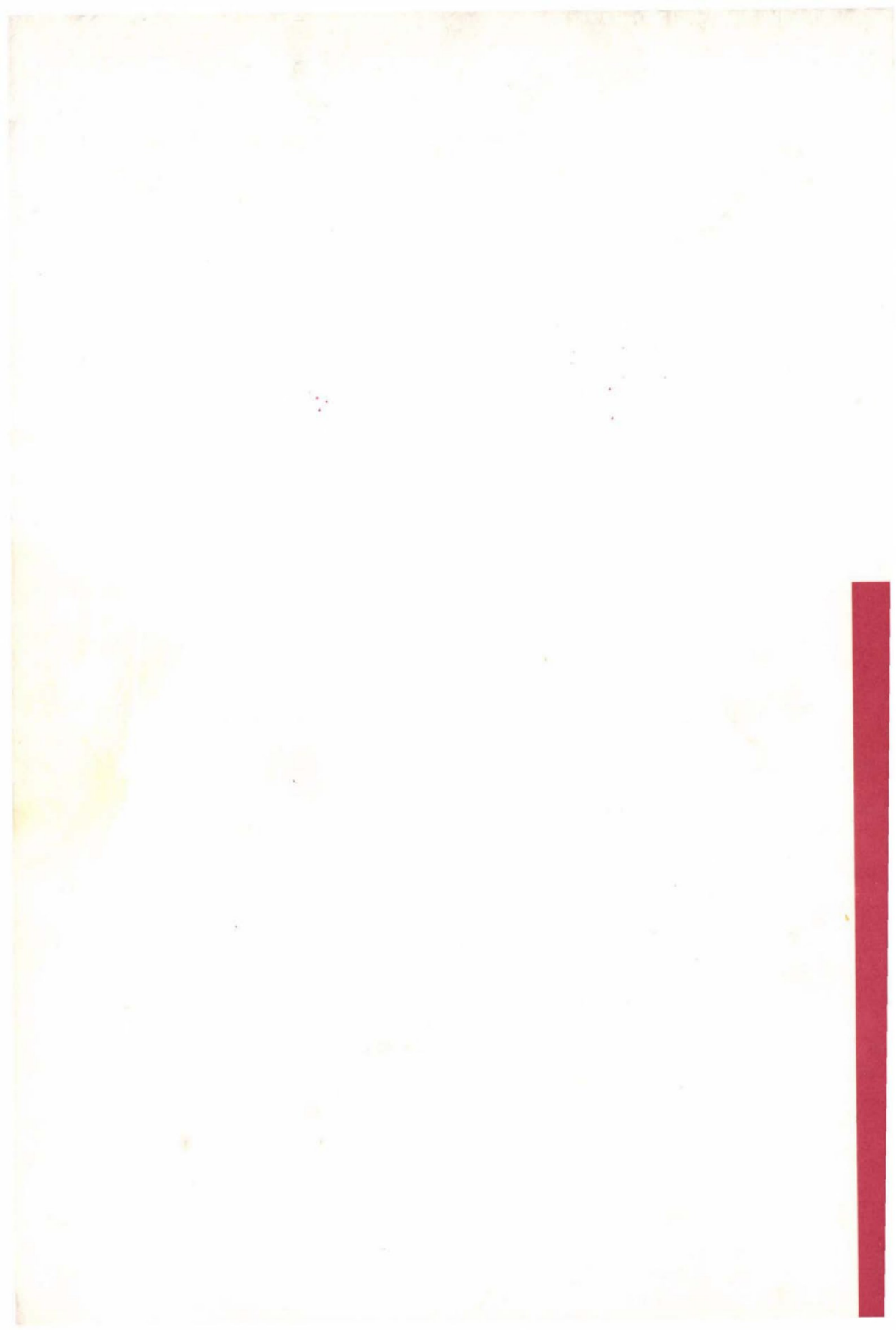
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MUCOSAL IMMUNITY

(A REVIEW)

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(Received September 3, 1993)

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Introduction

The mucosal surface of the host provides an excellent site for adhesion of a wide variety of microorganisms [1]. Soon after birth the mucosal surface of the upper respiratory tract, the gastrointestinal and lower urogenitally tract become colonized by a variety of bacteria and other microorganisms contained in inspired air, ingested food and fetal excretions. Most of these organisms become established as the indigenous microflora, the so-called normal flora. Other mucosal surfaces such as the lower respiratory, biliary and the upper urinary tracts are normally sterile in the healthy host. During the state of good health all of the mucosal surfaces provide remarkable barriers against attachment of invading bacterial pathogens. When these barriers break down, however, pathogenic bacteria quickly gain a foothold and colonize large areas of the mucosal surfaces that are normally sterile or occupied by the indigenous flora. From these colonized sites pathogenic bacteria produce infectious diseases either by invading deeper tissues or excreting toxins that damage local or distant tissues [2].

The pathogenesis of bacterial infectious diseases arising from mucosal surfaces involves a number of distinct interactions between the host and the bacterial pathogens. Virulence factors of the bacteria enable the organisms to attach, multiply and

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translocate on mucosal surfaces and evade the defence mechanism of the host, both on these surfaces and in deeper tissues. To counter the virulence factors of the invading bacteria, the host develop remarkable barriers including chemical and mechanical modalities as well as specific and non-specific, humoral and cellular elements. Among the mucosal defense mechanisms the most important non-specific factors on the oral mucosa are: amylase, lysozyme and lactoferrin [3] and on the gastrointestinal mucosa: the mucus, bacterial antagonism and desquamation [4]. While the bacteria need iron for their multiplication, the iron binding capacity of the secretory IgA (sIgA) is an important part of the defense mechanisms, too [5].

The most important part of the mucosal defense mechanisms is the specific humoral and cellular immune system. Studying fetal and postnatal parotid gland specimens Thrane et al. [3] found that in the former IgM and IgA, but not IgD and IgE producing cells were seen (ratio: IgM; IgA; IgD; IgG; IgE = 4; 1; 0; 0; 0).

The IgA subclass dominated and these were mostly J-chain positive. Only a small amount of secretory components (SC) were present in the fetal specimens. In the postnatal specimens SC increased markedly along with growing number of IgA and IgD producing cells (ratio: IgA; IgM; IgD; IgG; IgE approximately 4; 2; 1; 1; 0). The IgA subclasses remained predominant although the proportion of IgA₂ positive cells tended to be raised. Both types of cells were J-chain positive. The mucosal immunity in many respects are separate from the systemic immune system. According to Holmgren et al. [5] the intestine is the largest immunological organ in the body. It comprises 70–80% of the total immunoglobulin producing cells of the body. It produces together with parotid glands more secretory IgA (50–100 mg/kg body weight/day) than the total production of IgG in the body (ca. 30 mg/kg/day).

Overview of B cell differentiation

Each types of blood cell originate from common stem cell. The c-kit on the surface of these cells bind stem cell factor (SCF) in autocrine form and cooperating with local soluble factors (cytokines and colony stimulating factors) they become to lymphoid and myeloid progenitors [6]. In the mucosal immunity the IgA B cell differentiation is important. Its differentiation can be seen in Fig. 1. The earliest cell of the B cell series developed from lymphoid progenitor as an effect of IL-7, is characterized by bearing certain B cell specific surface determinants detected by monoclonal antibodies [8]. These early B cells develop into "pre-B cell" under the control of IL-4, which are cells that contain cytoplasmic μ -chain, but not whole IgM molecules: such cells bear neither surface immunoglobulins nor other mature B cell markers [9]. Pre-B cells develop into "immature B cells" by the help of IL-4 and IL-5 that are capable of synthesizing whole IgM molecules and that bear surface IgM [10]. Immature B cells in turn become "mature B cells" which synthesize both IgM and IgD and bear these immunoglobulins on their surface. Further development of B cells involves interactions of the cell with antigen as well as antigen non-specific factors derived from T cells (such as TGF- β) [6, 11, 12].

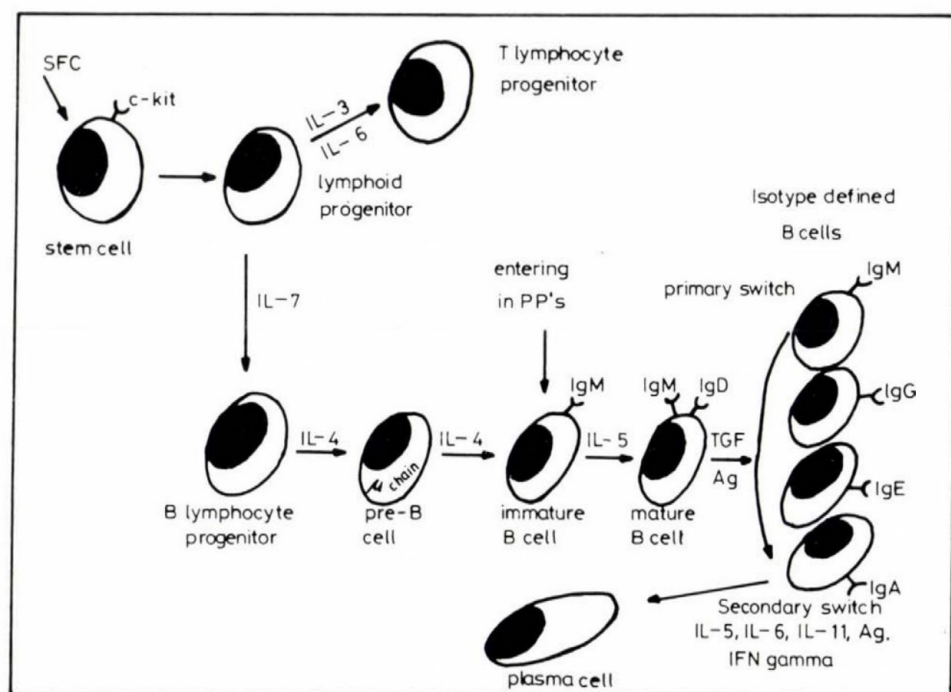


Fig. 1. Development of the B lymphoid cells and the isotypic differentiation

Such interactions initiate isotype differentiation process during which the B cells become definitive IgM, IgE, IgG or IgA cells [13]. Strober et al. [14] have found that sIgM bearing "virgin" B cells entering the Peyer's patches are subject to a microenvironment (most probably organ specific stromal cells), which brings about initial or primary IgA switch differentiation. For the reasons mentioned above this probably does not involve TGF-beta, which instead appears to rate on cells such as CH 12 LX B cell already undergone the initial steps of IgA isotype switching. The next stage of IgA B cell differentiation involves a cell which expressed sIgM and sIgA simultaneously and appears to produce C μ and C- α mRNA transcript in the absence of deletional rearrangement. Finally committed IgA B cells emerge from the dual bearing cell population, which express only sIgA. These cells can migrate out of Peyer's patches and respond to various terminal differentiation factors such as IL-5, IL-6 and interferon gamma. In the last decade it has become clear that these cellular transformations reflect genomic events that result in massive rearrangement of genes encoding the heavy (H) and light (L) chains of the immunoglobulin molecule [15]. Prior to differentiation the region of the genom dedicated to the Ig molecule consist of many structural genes on a relatively long stretch of DNA [6]. In the case of the variable domain of heavy chain these structural genes consist of several hundreds

variable region (V-region) genes, followed approximately 20 diversity (D-region) genes and four joining-region (J-region) genes. These J-region genes are followed by constant domain (C-region) genes arranged on the DNA strand in fixed order. In the mouse this consist first of μ and delta C-region genes then the various gamma subclass C-region genes and finally the epsilon and alfa C-region genes [16] (Fig. 2). In the human this basic arrangement has undergone gene group duplication in that we find the μ and delta C-region genes followed by two tandem segments each containing gamma, epsilon and alfa genes. This first segment is composed of γ_3 and γ_1 genes followed by non-expressed epsilon gene and the alfa genes. The second segment is composed of γ_3 and γ_1 genes followed by an expressed epsilon and α_2 gene [17]. The earliest step in the process leading to functionally active immunoglobulin gene, involves rearrangement of the DNA that controls the V region of the Ig molecule. During this step, one of the V region genes and one of the J region genes undergo a molecular mechanism that involves the looping out and excision of DNA intervening between the retained genes [18]. Variability in the sequencing of resultant V, D and J genes and the occurrence of somatic mutations lead to diversity in the assembled V region gene and this explain why each cell is virtually unique with respect to antigenic specificity [16]. Genes encoding the light chain V region undergo similar rearrangement but as mentioned in this case there are no D genes on the relevant DNA strand. The B cell isotype differentiation can be seen in Fig. 2. According to Erhardt et al. [19] TGF-beta has been reported to play an important role in IgA isotype expression when B cells are stimulated with LPS. This TGF-beta caused a small but significant absolute increase in the IgA secretion; these effects were enhanced by IL-2 and IL-5. TGF-beta provides only a partial or incomplete IgA switch signal, but additional (T cell dependent and/or independent) factors are involved in IgA isotype switching and differentiation.

Cellular migration in the mucosal immune system

The elaborate regulatory system controlling IgA responses would be too little avail if there did not exist a mechanism for focusing IgA B cells at mucosal sites. It is clear that such a mechanism does exist, by which cells developing in organized mucosal lymphoid tissues (such as PP's, MALT, GALT) and migrating out of these tissues have a strong tendency to return to the diffuse mucosal lymphoid tissues, the areas underlying the mucosal epithelium. In initial studies aiming at the cellular localization or homing in the mucosal immune system, the fate of injected blast cells marked with DNA labels (^3H -thymidin, ^{125}I -iododeoxyuridin) was followed autoradiographically or direct tissue counting. These studies showed that blast cells obtained from thoracic duct lymph or from mesenteric lymph nodes localized in the gastrointestinal [19] mucosa, whereas cells obtained from peripheral nodes, did not [20, 21]. Cells taken from bronchial nodes also had a tendency to localize in mucosal areas, but here the localization pattern weighted toward lung mucosa [22].

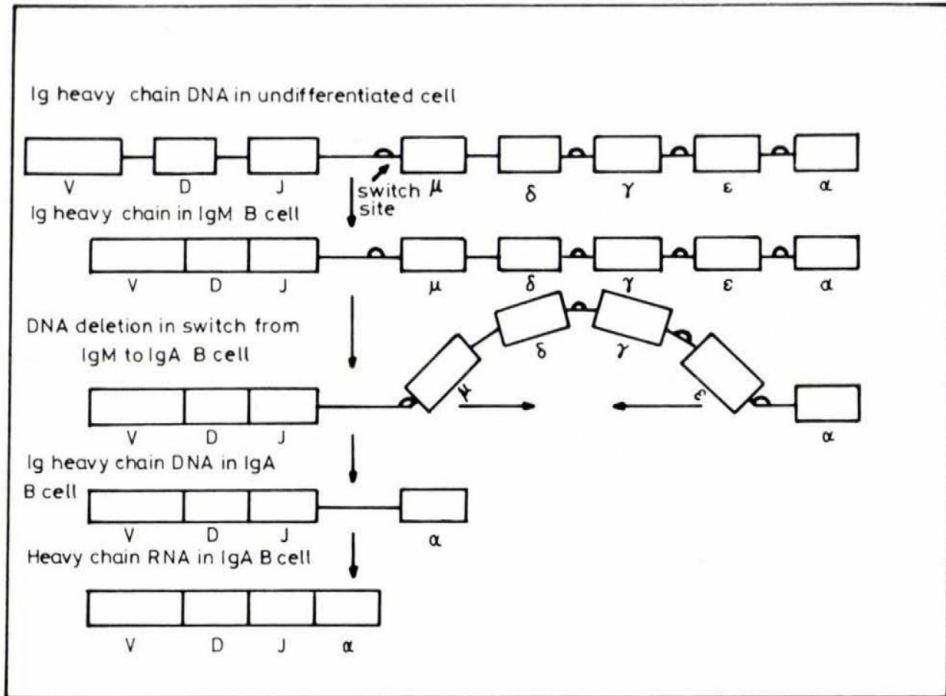


Fig. 2. Structure of genome containing Ig encoding genes. As explained in the text the rearranged V.D.J. regions encode for the V-region of the Ig Molecule. Genes encoding for various heavy chain C regions are arranged in sequence and undergo deletion during isotype differentiation. Such deletion involves pairing of switch sites 5' to C region genes and deletion of the resulting loop. It is seen that IgA C-region-encoding gene is the most 3' of the various C region genes

It was found that the majority (70–90%) of the migrating blast cells are B cells, most of which express IgA, however, if the cells have arisen in the bronchial lymphoid follicles, the migrating B cell blast population may contain a significant number of IgG B cells [23]. This might indicate that T cell blasts are less restricted to mucosal areas than B cell blasts. T cells as opposed to B cells settle in two distinct sites in the diffuse mucosal areas: in the lamina propria per se and between epithelial cells. The latter cells constitute a distinct cell population known as the intraepithelial lymphocytes (IEL). IEL-s are considered to be major effector cells in mucosal immunity. In an experiment where orally given sheep red blood cells tolerated rat lymphocytes were studied, it has been found that alpha-beta TCR-CD 3+ IEL-s possessed immunoregulatory functions [24]. According to Beagly and Elson [25] IEL consist mainly CD 8+ T cells. They have been shown to produce interferon-gamma. A certain assortment of cytokines are greatly increased in inflammation. This assortment including IL-1, IL-6 and IL-8 elevated in a wide variety of chronic inflammatory states in oral tissues as well. The Leu 8 membrane lipoprotein is the primate homologue of the murine MEL 14 peripheral lymph node homing receptor and is expressed on a majority of circulating

lymphocytes but only on a few lymphocytes in the intestinal lamina propria [26]. Cross linking of the Leu 8 (Selectin, LECAM-1) on both T and B cells results in modification of cell function. The Leu 8 participates in the activation of T cells probably via its association with TCR/CD 3 complex. This means that Leu 8 have either homing or activating functions [27].

Cellular migration and function within the mucosal immune system

The migratory pattern of cells within the mucosal immune system can consist of a pattern generalized to all mucosal areas or patterns that are organ specific. Thus we find that cells sensitized in bronchial lymphoid tissue have a predilection for respiratory mucosae, whereas cells sensitized in gastrointestinal lymphoid follicles favour gastrointestinal mucosa as their homing destination [28], (Fig. 3). In addition there even appear to be suborgan circulatory patterns in that cells originating in the colon have a greater tendency to return to the colon, than to other parts of the gastrointestinal tract [29]. This suborgan circulation may be advantageous to the individual because it ensures that cells sensitized to a particular antigen will accumulate (or expand) at sites where that antigen is ultimately found.

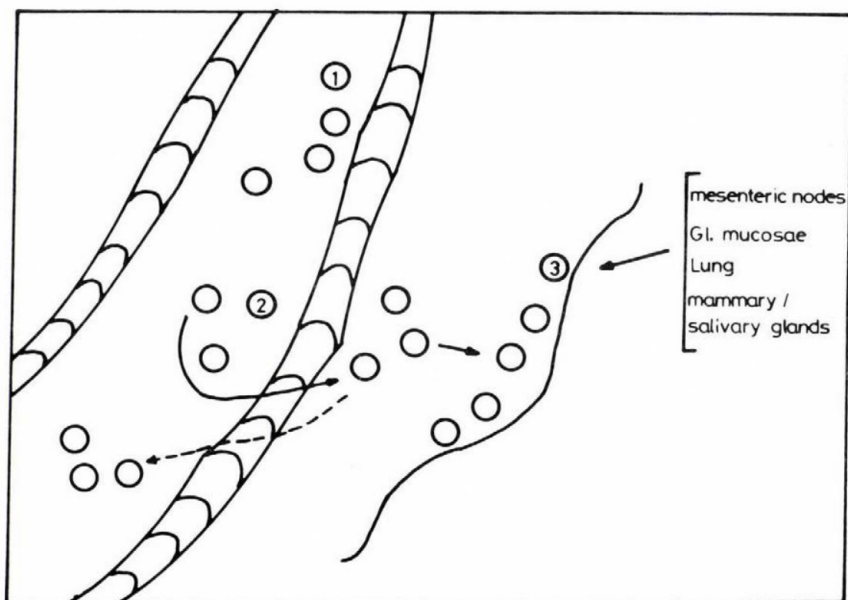


Fig. 3. Factors involved in homing of mucosal cells to mucosal sites: (1) circulation via blood and lymph; (2) efflux of migrating PP' cells based on an interaction with the vascular endothelium; (3) interaction with antigen at local sites

Not all intraepithelial cells are T cells and many may not even be lymphocytes. For instance one population of intraepithelial cells are granulated cells that can be induced by appropriate stimuli to secrete mediators characteristic of mast cells [30]. These cells which are also found in the lamina propria, appear to be derived from non-T cells following exposure to T cell factors [31]. Undoubtedly this is the population that is a greatly expanded during mucosal helminth infections. In this instance it can be postulated that the parasite initially cause T cell stimulation and thus the release of mast cell mediated cytokines.

The latter acts on mast cell precursor in the IEL population and in the lamina propria to induce maturation of large numbers of mucosal mast cells [32, 33]. A second intraepithelial cell population has natural killer (NK) activity. In this regard Tagliabue et al. [34] have shown that there exist within IEL large granular lymphocytes with clear cut NK function. Cells in the IEL may play a critical role in both health and diseases. For example the NK cells in this population may constitute a first line of defense for elimination of virus infected host epithelial cells. In addition inasmuch as IEL numbers are increased in various mucosal diseases [35] IEL may be integral components of certain disease mechanisms.

Some humoral aspects of mucosal immunity

It has been known for nearly 25 years [36] that IgA is the predominant class of immunoglobulins in various mucosal secretions. Following the original description of predominance of IgA plasma cells at mucosal sites, a variety of studies have quantitated the classes of Ig containing cells in various secretory as well as peripheral lymphoid organs [37, 38]. Cargo et al. [39] have shown that human spleen, tonsil, bone marrow and peripheral lymphoid tissues contain predominantly IgA₁ staining cells (75–90%), whereas the small and large intestine, salivary and lacrimal glands exhibit approximately equal numbers of IgA₁ and IgA₂ plasma cells. A large number of cells staining for J-chain have been found in mucosal tissues and of the IgA₂ cells contained J-chain in a higher percentage than did IgA₁ positive cells. Thus it would appear that the proportion of IgA₁ and IgA₂ molecules in serum secretion is similar to the tissue distribution of the cells containing the two subclasses. Recent studies [40] suggest that less than 2% of the total salivary IgA originates from plasma.

The most important part in mucosal immunity is played by the secretory immunoglobulins. Secretory IgA (sIgA) is an 115 molecule (MW 390 kD) consisting of two IgA monomers covalently bound by joining chain (J-chain) and complexed to one molecule of SC. J-chain has a molecular weight of 15.6 kD and is covalently associated with polymeric immunoglobulins (IgA and IgM) [41–43]. A single J-chain is present per mole of polymeric Ig regardless of the polymer size (dimer, trimer, tetramer or pentamer). The carbohydrate portion of J-chain consists of a single asparagine-linked oligosaccharide composing approximately 7.5% of its molecular weight. The primary structure of the polypeptide portion of the J-chain has been elucidated [45]. Analysis of the interaction of monomer Ig to form polymers in vitro suggests that the J-chain is probably involved in the initiation of polymerization. In IgM and higher polymers of IgA J-chain binds via disulphid linkage to the penultimate cystein residue of the two

monomer heavy chains. Apparently this association with J-chain results in a dimer conformation that tends to promote interaction with additional monomeric subunits giving rise to higher-order IgA polymers and the pentameric IgM molecule [41, 46]. Little J-chain is present in unstimulated B cells, but following exposure to mitogen or antigen there is a marked increase (100-200 fold) in intracellular J-chain [47]. There is some evidence that the J-chain is rapidly degraded in cells producing monomeric Ig [48]. Genetic analysis [49] indicates that there is a single J-chain gene in the mouse haploid genome located in chromosome 5, that is unlinked to any of the Ig genes. The same gene product must therefore be used for polymerization in both IgM and IgA. The locations of genes for J-chain and Heavy (H) and Light (L) chains on different chromosomes suggest that activation of polymeric Ig production in B cells is mediated by a mechanism that can generate either three separate signals or one signal that subsequently affects loci on the other chromosomes. It should be emphasized that although polymers lacking J-chain [50] are capable of binding secretory components, they do it in smaller amounts and with lesser affinity than those containing J-chain. Thus the conformation induced by J-chain may be optimal to the subsequent binding of SC.

The secretory component is a glycoprotein of approximately 80 kD that is synthesized in epithelial cells of mucous membrane [51, 52]. Human milk SC contains 23.4% carbohydrates [53]. In addition to bound SC (in IgA and IgM), SC is also present in a free or unbound form in secretions from the digestive, respiratory and other exocrine glands [54]. By immunohistochemical localization, SC is found predominantly, but not exclusively in serous cells of epithelial and exocrine glands. It is also present in certain ductal epithelial cells, including those of salivary, bile and pancreatic ducts. The number of measurable IgA dimer binding sites per epithelial cells varies (260-7000 sites per mammary cells) and is directly related to the number of unoccupied receptor (SC) molecule in the membrane [55]. The mucosal tissues are defended by a local immune system with properties and functions that in many respects are separate from the systemic immune system.

Transport and distribution of IgA antibodies

IgA moves from one body compartment to another by a highly specific transport mechanism that allows for an orderly distribution of antibodies into those secretions where they are required. As already mentioned, transcellular passage involves the direct participation of SC, a protein synthesized by many mucosal epithelial cells that binds specifically to the Fc region of polymer IgA and IgM. SC is asymmetrically distributed in the membranes of epithelial cells in such a way that binding of IgA is followed by an orderly vectorial transport of the antibody into secretions and apparently preventing its encounter with degradative organelles e.g. lysozyme. Sullivan and Wira [56] have shown that movement of IgA across the cells may be modified by hormones. This studies in rats have shown that oestrogen administration increases the accumulation of polymeric IgA in uterine secretions, possibly mediated by increases in membrane SC.

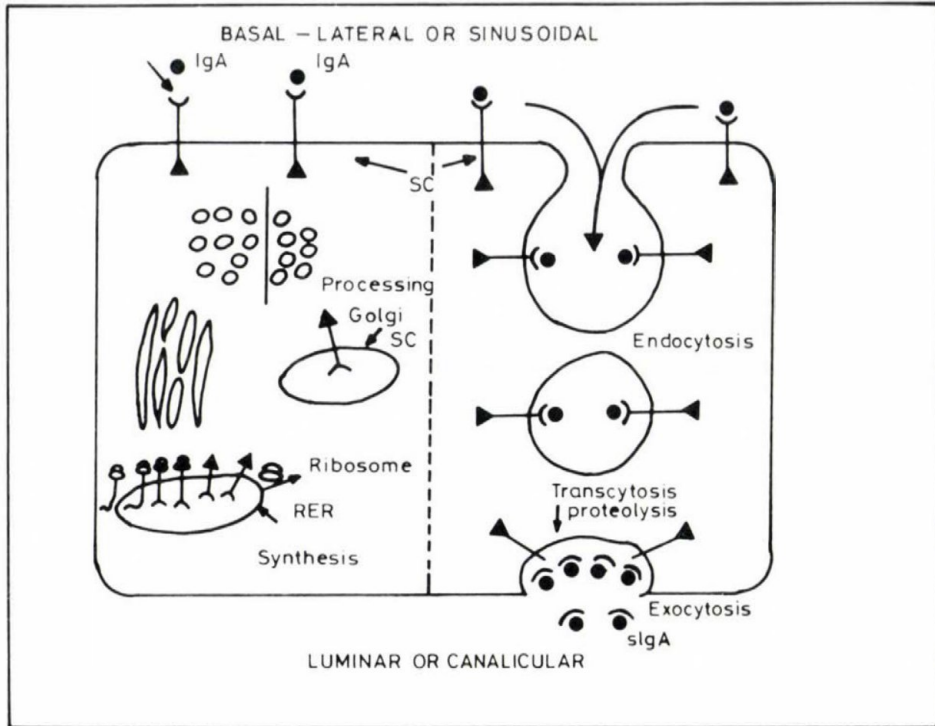


Fig. 4. Model for transport of IgA and IgM across the mucosal epithelial cells. The synthesis of SC (left side) and the steps in the transport of Ig across the cell (right side) are graphically represented. The transmembrane SC (—) consists of three domains (a) cytoplasmic (▴); (b) membrane-spanning (—) and (c) ectoplasmic (◁). The ectoplasmic domain acts as the external receptor for J-chain containing polymeric Ig-s (●)

In another point of view Alverdy and Aoy [4] found that glucocorticoids may promote bacterial translocation by impairment of mucosal IgA synthesis. Comparison of SC isolated from deoxycholate-solubilized plasma membrane from rabbit liver and mammary gland have revealed a heterogeneous population of molecules in two size ranges: one group with a molecular weight of approximately 100 kD and the other 80 kD. This raises the possibility that a portion of SC occurs as a large transmembrane from anchored in the lipid bilayer of the membrane. Mestov et al. [57, 58] have identified transmembrane and secreted form of SC using a cell-free translation system supplemented with dog microsomal membrane. Studies of the structure of SC have been extended by analysis of human adenocarcinoma cell line (HT-29) that synthesize SC. Huang et al. [59] demonstrated that HT-29 cells have a polarity and are capable of binding dimer IgA at the basal and lateral plasma membranes and translocating IgA across the cell to the luminal membrane. Figure 4 represents a hypothetical model for the transport pathway of IgA across the epithelial cells. SC is synthesized on

polysomes as a transmembrane protein that is integrated into the rough endoplasmic reticulum (RER), with its ectoplasmic domain projecting into the lumen of the RER. After transport to the Golgi (where terminal saccharide units are added), SC is inserted into the basal-lateral region of the epithelial cell membrane. The ectoplasmic domain of the transmembrane [60] protein project from the cell surface and acts as a receptor for polymeric immunoglobulins. After complexing with J-chain-containing polymers (IgA and IgM), endocytosis is initiated and Ig is transported across the cell within vesicles that ultimately fuse with the apical plasma membrane.

Whereas colchicine has little effect on binding and internationalization of IgA [61], can move across the cell. There are no data suggesting fusion of these vesicles with lysosomes, but this has not been excluded. Somewhere along this transcytotic process the transmembrane Ig complex is proteolytically cleaved, releasing soluble sIgA and leaving behind the residual membrane protected and cytoplasmic domains. The location and characteristic of the proteolytic enzyme that is responsible for this cleavage may be highly specific. The SC attached to IgA as well as free SC is of 80 kD and therefore the portion remaining in the cell is approximately 20 kD. Most of the residual SC represents unusually large intracytoplasmic domain (compared with other transmembrane proteins). This scheme is consistent with previous fluorescence studies localizing SC and IgA to identical regions of the basal-lateral plasma membrane of intestinal and salivary gland cells and is consistent with the finding of IgA and SC in membrane bound intracytoplasmic vesicles [60, 62–64]. The entire SC receptor consists of 772 amino acids (aa) and includes: an 18 amino acid signal peptide, a 629 amino acid ectoplasmic domain, a 23 amino acid (largely hydrophobic) membrane-spanning segment, and a 103 amino acid cytoplasmic tail. A sixth domain is somewhat more distantly related and includes the membrane-spanning portion. When using the Dayhoft Protein Data Bank this was compared with other receptors it was suggested that these proteins belong to the immunoglobulin superfamily. The most significant homologies were found between IgD V-regions (identical amino acids as on average 28%, and when compared on the basis of similar side chain, the homology increased to 56%). A striking feature was a highly conserved 18 residue consensus sequence that was found in each of the five domains and closely resembled a segment that had been reported [65] to the primordial building of immunoglobulin heavy-chain available region. The evolution of a transport system in which a proportion of the receptor remains attached to the ligand may be beneficially in the immunoglobulin system, because SC appears to protect the Ig molecule (a) from degradation in its passage through the cell and (b) from the mucosal contents once SC reaches the lumen. In addition sIgA may interact with epithelial ductal cells and/or the mucosal blanket on the surface of the epithelium, and it has been suggested [66, 67] that IgA antibodies coating the luminal surfaces of epithelia act as a barrier to the entrance of antigen.

Antigen uptake and processing in the intestinal tract

Most macromolecular absorption (including soluble proteins and particular matter) probably occurs via "M" cells (they can be found in the PP's lymphoid follicles) as shown by the work of Backmann and Cooper [16] involving ferritin tracers in animals and the electron microscopic studies of Owen and Jones [68] in humans. It has also been shown that uptake of antigenic material in the adult is modulated by antibody [66]. It has not been excluded that several cell types that have been implicated in other tissues may be involved in antigenic presentation, including Ia positive macrophages [69], dendritic cells [70] and B cells. Lipopolysaccharides (LPS) that are present in the gut of conventionally fed mice have a dramatic effect on the activation of B cells for the presentation of antigen [71].

Effector functions of mucosal antibodies

The effector functions of mucosal antibodies, practically IgA, have been debated for some years [72-74]. Both IgA and IgM have properties that ensure efficient translocation across epithelial membranes and these are centres to their action.

IgA antibodies are largely silent as mediate on inflammatory reactions. This is particularly notable when IgA is compared with IgG and IgM antibodies of similar specificity that participate in an array of inflammatory functions, such as complement activation, neutrophil chemotaxis and phagocytosis [72, 74]. It is now recognized that IgA mediates an "escort" function involving efficient disposal of antigens through a mechanism of inhibition of adherence or transport from one body compartment to another. The mucosal immune system is a part of a comprehensive local systemic defense mechanism in which IgA plays a major role by disposing of microbial and dietary antigens locally and/or preventing them from entering the body.

Neither secretory nor serum IgA monomers or dimers activate complement in vivo. Several researchers have described complement activation by IgA in vitro [75-78], but these experiments typically involved aggregation or proteolytic disassembly of the protein. Serum IgA blocks complement mediated effector mechanisms [79] and it has been suggested that it functions as an antiinflammatory Ig in diseases such as nephropathy, dermatitis herpetiformis, and different kinds of oral aphthous ulcers involving tissue deposition of IgA autoantibodies or immune complexes, when either complement components are absent or associated deposits of complement activating IgG or IgM can be demonstrated. At present there is no definite evidence that IgA can mediate immunologic tissue damage through complement dependent mechanisms. Van Epps et al. [80] reported that using a modified Boyden chamber technique polymeric IgA paraproteins inhibited chemotaxis of neutrophils and eosinophils [81]; and these proteins also suppressed the bactericidal activity of neutrophils without interfering with cell metabolism, membrane fluidity or the ability of the neutrophils to participate in the lysis in the IgG coated avian erythrocytes in ADCC system [82]. Fanger et al. [84] reported that although circulating human neutrophils were unable to phagocytose erythrocytes sensitized by specific rabbit IgA antibodies, neutrophils recovered from mucosal sites, such a gingival crevices of teeth

were able to phagocytose such cells. They also demonstrated that more gingival neutrophils (62%) than peripheral blood neutrophils (35%) had IgA receptors. The presence of IgA evoked both an increase in the number of IgA receptors per neutrophil and an increase in the overall number of receptor positive cells. The simultaneous sensitization of erythrocytes with IgA and IgG lowered the requirement of IgG needed to promote phagocytosis. A recent study [85] demonstrated an in vitro cell mediated antibacterial effect of GALT (gut associated lymphoid tissue) lymphocytes against *Shigella* X 16 (a hybrid strain between *Shigella flexneri* and *Escherichia coli*). Murine lymphocytes from GALT and MALT (mucosal associated lymphoid tissue), but not from other tissues (thymus, or popliteal lymph nodes) exerted natural antibacterial activity. The type of cell involved was not defined but some evidence was presented that null or K lymphocyte (not macrophage) was mediating the ADCC by sIgA. If this work was verified, it would represent an important new role for sIgA in protecting the host against infectious agents of mucosal level. IgA antibodies have been shown to have several effector functions that are not dependent on phagocytic cells, lymphocytes and complement: also these biological properties may be of crucial importance in defence against mucosal pathogens and undesirable gastrointestinal absorption of ingested antigenic materials. Most of these are known and have been extensively reviewed [86]. Mucosal IgA neutralizes viruses, as exemplified by the extensive studies of Orga et al. [87] on poliovirus immunization by the oral route. In the case of bacterial infections, IgA blocks the attachment of pathogens to relevant mucosal tissue and cells and such an adherence inhibitory function has been demonstrated in several experimental models and in humans, particularly with reference to the oral microflora [88], urinary tract pathogens and *E. coli* and *Vibrio cholerae* as intestinal pathogens [89].

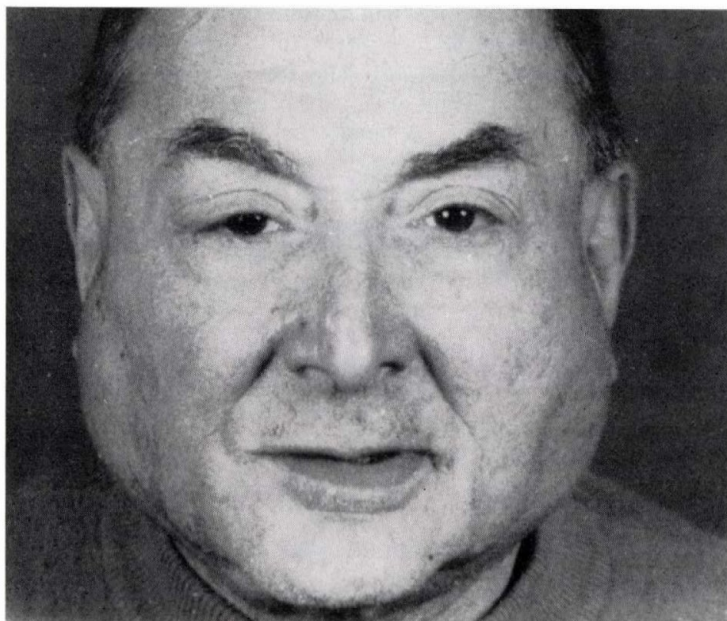


Fig. 5. (1) Bilateral parotid gland enlargement in a patient with primary Sjögren's syndrome

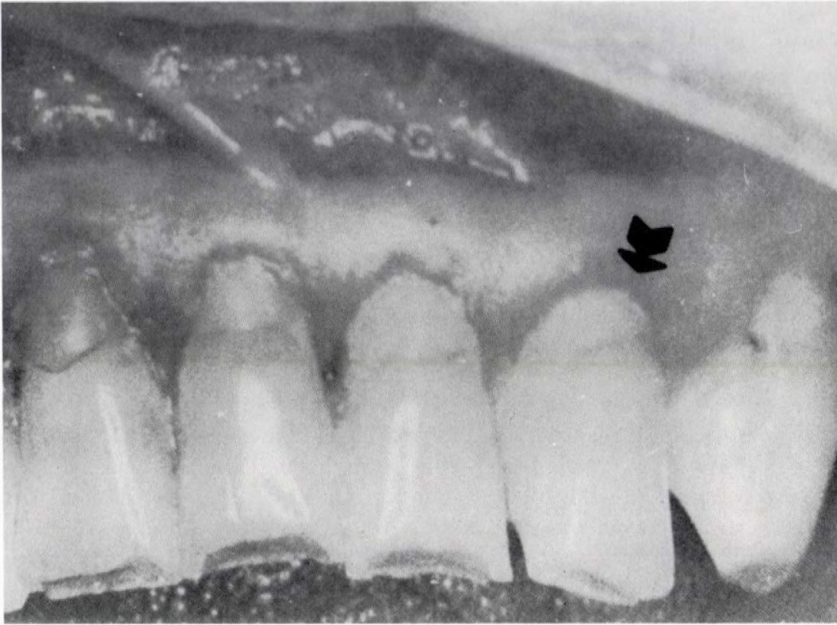


Fig. 5. (2) Pattern of cervical caries which is suggestive of underlying xerostomia

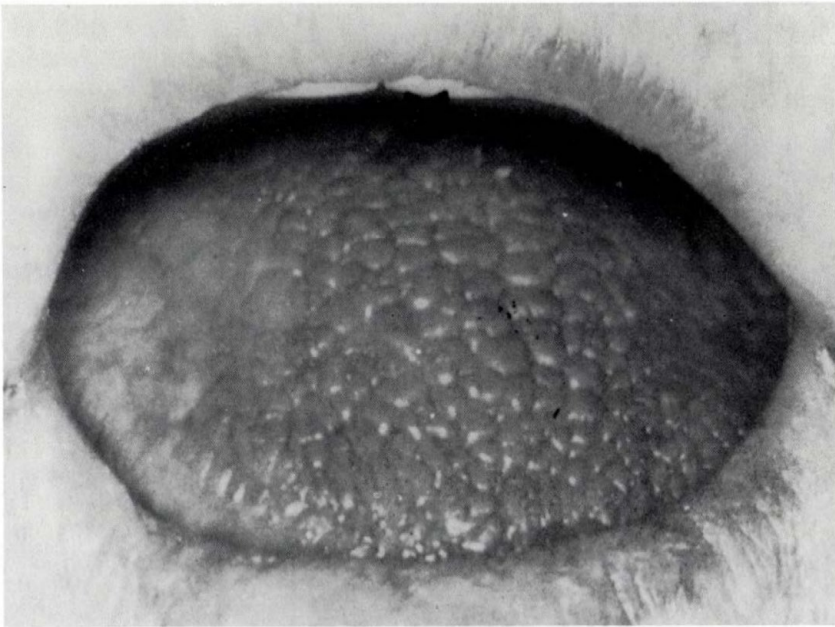


Fig. 5. (3) Obvious xerostomia and a lobulated appearance on the tongue in a patient with Sjögren's syndrome

Another biologically relevant function of mucosal antibodies is the binding and subsequent inhibition of absorption of soluble macromolecular antigens (immune exclusion) as shown in extensive studies by Walker and Bloch [66]. Everted gut sacs of rats orally immunized with BSA or horseradish peroxidase showed an antigen specific reduction in passage of the immunizing antigen to several surfaces. The mechanism was thought to involve immune complex formation followed by subsequent rapid degradation of these complexes by proteolytic enzymes of pancreatic origin. A clinical counterpart of immune exclusion may exist in IgA deficient individuals who have high levels of serum antibody to food antigens, particularly bovine milk proteins. Cunningham-Rundles [91] has identified dietary proteins in circulating immune-complexes in such individuals, presumably because they experience chronic hyperabsorption of macromolecules that remain antigenically intact. An interesting recent observation [92] is that a small but significant fraction of the immune-complexes in IgA deficient serum involves idiotype-antiidiotype interactions. Because antiidiotype antibodies have been shown to have both suppressive and stimulatory activity on idiotype production and both idiotype and antiidiotype Ts have been defined, the foregoing findings have potential implications for regulation of immune reactivity to environmental antigens.

In some oral diseases arise problems associated with the suppression or activation of mucosal immunity. Most of them are autoimmune or infectious diseases [93]. The most characteristic disease in this group is Sjögren's syndrome in which the autoantibodies are produced against the strial duct of the parotid gland. In the early stage of the disease SC, IgA and the quantity of saliva decreases. These symptoms causes very unpleasant oral burning sensation and then dramatic increase in the caries frequency and in some cases ulceration on the mucosal surface (Fig. 5/3). Similar but less serious diseases are the oral aphthous ulcers [94] including aphthous ulcers (Mikulicz's), herpetiform ulceration (Cooke's) and periadenitis mucosae oris (Sutton's). These self-generating diseases appear on the oral mucosa more and more frequently (Figs 6, 7, 8) [95]. Among the oral infections the most frequent is the herpetic infection caused by the herpes simplex virus (HSV). The primary infection is herpetic gingivostomatitis, which is a serious disease in childhood. After the primary infection the infectious agent can be found in the ganglions of facial and trigeminal nerves as a slow or latent virus from where the virus can spread and causes the secondary diseases such as nasal and labial herpeses. The primary HSV infection can become recurrent in the case of immunodeficiency [96] (Figs 9, 10). Also a frequent oral mucosal disease is the erythema multiforme, which is an allergic disease caused by pollenic (inspired) or drug (ingested) antigens.



Fig. 6. Aphthous ulcer (Mikulicz's)

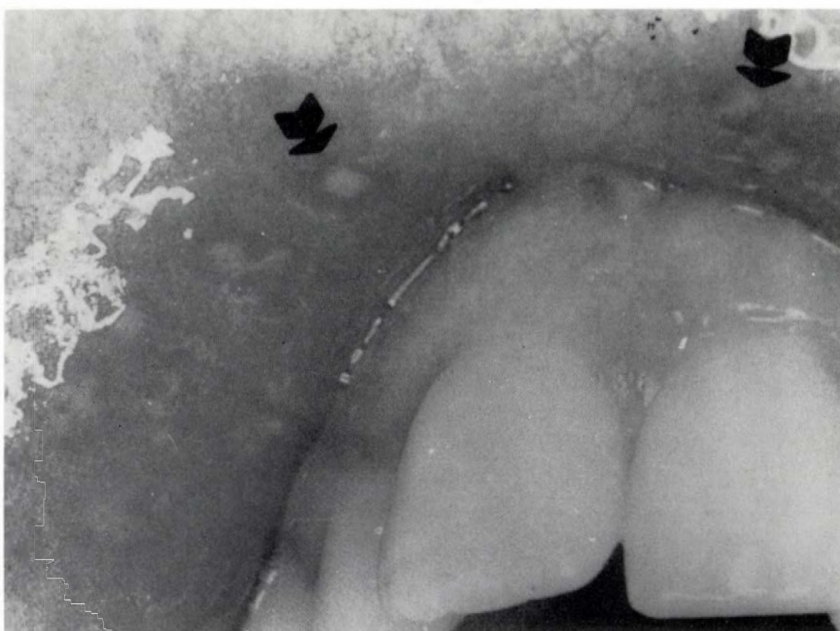


Fig. 7. Herpetiform ulceration (Cooke's)

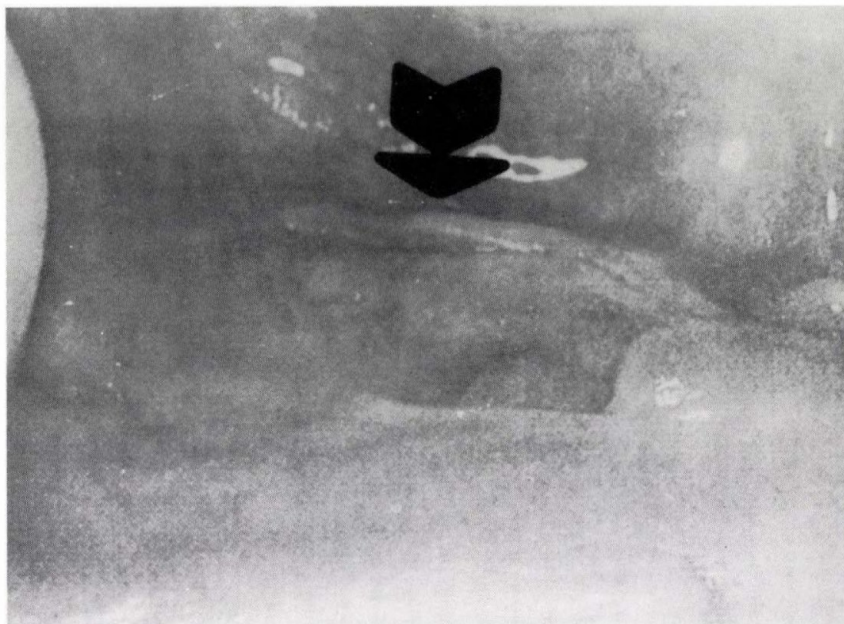


Fig. 8. Peradenitis mucosae oris (Sutton's)

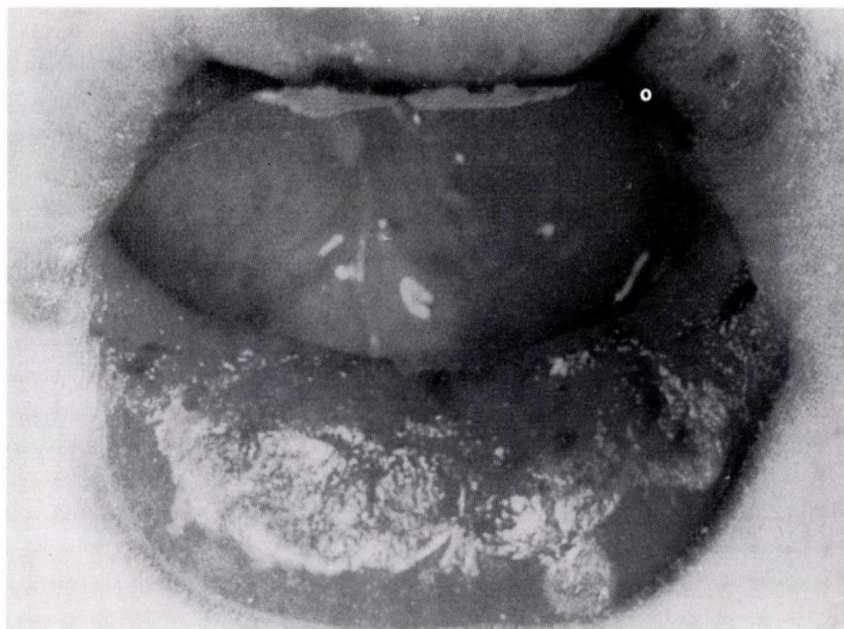


Fig. 9. Extensive oral ulceration of primary herpetic gingivostomatitis



Fig. 10. Early herpes labialis

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EFFECT OF PERMANENT STARVATION ON THE INSULIN RECEPTORS OF THE NUCLEAR ENVELOPE OF *TETRAHYMENA*

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Insulin receptors of *Tetrahymena* following a two-hour starvation (one cell generation) can bind the insulin similarly to the fed, control cells. The nuclear envelope of *Tetrahymena* cells which have already met the hormone (imprinted cells) can bind the insulin significantly stronger than the control. This binding capacity has a mild decrease after a two-hour starvation; after 18-hour starvation the decreasing effect is more expressed and reaches the control level. On the ground of the experiments it is probable that the insulin receptors of the plasma membrane and the nuclear envelope are identical.

In the plasma membrane of the unicellular organisms there are structures which are able to recognize substances of the environment [1]. When a signal molecule (hormone) is present in the environment, it is able to fasten the more or less specific membrane structures (hormonal imprinting). The result is that the binding site turns into specific receptor [2, 3]. The structures of the membrane developed before are transmitted to the progeny generations with a mechanism which is not yet understood exactly. This way the binding capacity of these receptors differ to those which have never met the hormone, and this altered responsiveness can be detected in the following hundreds of generations [4].

In cells of the higher level organisms the receptors characteristic to the plasma membrane were demonstrated in the nuclear envelope, too [5–13]. Our previous experiments showed that the receptors of the nuclear envelope are also present in *Tetrahymena*, they can bind the hormone and are imprintable like the receptors of the plasma membrane [14]. These earlier experiments indicated that beside the identity of the binding capacity, there were altered reactions provoked by certain non-physiological influences on the nuclear envelope receptors as compared to ones of the plasma membrane. Chloroquine is a lysosomotrop substance and, being able to influence the internalization of materials into the cells, can inhibit the imprinting of the plasma membrane but not of the nuclear envelope [15]. These were the reasons why it is presumable that the physiological processes will also influence the binding capacity

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of the nuclear envelope less than the plasma membrane. In our present experiments we studied the effects of a strong however physiological process, the starvation.

Materials and methods

Strain. The investigated cells of *Tetrahymena pyriformis* GL were cultured at 28 °C in a medium containing 0.1% yeast extract and 1% Bacto tryptone (Difco, Michigan, USA). Cells of 24-hour-old cultures were used, the maximal cell density was 2×10^5 cell/ml.

Cultures of *Tetrahymena* cells. (1) Untreated culture of *Tetrahymena* cells. (2) 10^{-6} M insulin (Semilente, MC Novo, Copenhagen, Denmark) pretreated (imprinted) culture. After the pretreatment the cells were transferred into fresh culture medium for 24 h. (3) Cells of a culture identical to the control group were starving in Losina-Losinsky solution without shaking for 2 h. (4) Cells pretreated with 10^{-6} M insulin (identical to the imprinted group) were starving in Losina-Losinsky solution for 2 h. (5) Cells pretreated with 10^{-6} M insulin (identical to the imprinted group) were starving in Losina-Losinsky solution for 18 h (Losina-Losinsky solution: 0.011% NaCl, 0.01% $MgCl_2$, 0.001% $CaCl_2$, 0.02% $NaHCO_3$).

Isolation of nuclei. Cells of *T. pyriformis* cultured at room temperature were centrifuged and washed with 10 mM NaCl then homogenized in a dedicated buffer (0.04 v/v sodium deoxycolat, 0.04 v/v NP_4O , 6 mM $CaCl_2$, 37.5 mM NaCl, 1.5 mM Na_2HPO_3 , 250 µg/ml spermidine tetrahydrochloride, pH 7.6) for homogenization [16]. Under these conditions the recovery of cells was 90% without damage of the nucleus. After a 10 min rest, the solution containing cells and the buffer was centrifuged once more. The cells were suspended and treated with a Potter hand-homogenizer five times. It was centrifuged at 3000 g, then the pellet was washed with a solution containing 6 mM $CaCl_2$ and 0.28 M raffinose. The nuclei were separated on a "saccharose pillow" (2.14 mol/1.75 M saccharose) by ultracentrifugation (VAC 602) at 1500 g for 30 min. The nuclei were suspended in a rinse-buffer (0.25 M saccharose, 10 mM $MgCl_2$, 10 mM Na acetate pH 7.6). All steps of isolation were carried out on 0 °C.

Preparation of FITC-insulin binding assay on isolated nuclei. A concentration course of insulin (Semilente MC, Novo, Copenhagen) was diluted in incubation medium between 10^{-10} M and 10^{-6} M. The nuclei were fixed in the rinse-buffer containing 4% formaldehyde. The appropriate amount of insulin and FITC insulin (FITC-protein ratio 0.3%; protein concentration 0.21 mg/ml) were added simultaneously. This was followed by one hour incubation at room temperature. The nuclei were washed twice with the rinse-buffer, and once with PBS and distilled water. Samples were dropped onto slides and dried. In each group those nuclei served as absolute control which had the same proceeding but received "empty" rinse-buffer instead of insulin.

Measurement of FITC-insulin binding. The intensity of fluorescence was measured with a Zeiss Fluoval cytofluorimeter connected to a digital processor. This processor transformed the analogue signals to digital ones and they were registered by a Hewlett Packard HP 41CX calculator. In each group the intensity of fluorescence was measured on 20 nuclei, the experiment was carried out in 5 replicas. Each point of the figures represents the average value of 100 nuclei.

Statistical analysis. Data were treated with the Student's *t*-test and analysis of variance by the help of Harward program and IBM AT computer.

Results and discussion

The individual life-span of *Tetrahymena* is about 2 h. This was the reason that we chose the 2 h starving period in our experiments. The results clearly show that starvation for this period does not influence the binding capacity of the nuclei (Fig. 1)

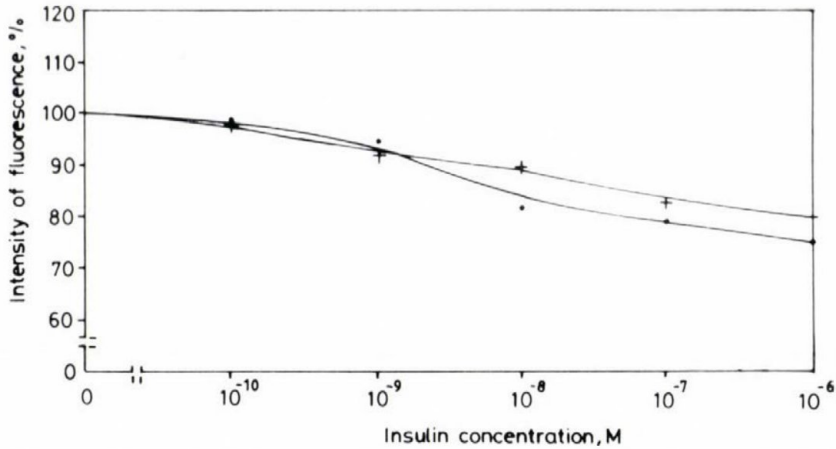


Fig. 1. Displacement of insulin binding of control (-) and 2 h starved (+) *Tetrahymena* cells in the presence of unlabelled insulin. Values are related to the prevailing controls as 100%

and the result of displacement (presence of unlabelled insulin) shows the same as in the controls.

As it was concluded from our previous works [17], the binding value of insulin pretreated (imprinted) *Tetrahymena* cells (between 10^{-8} and 10^{-6} M) differed significantly from the untreated control (Fig. 2).

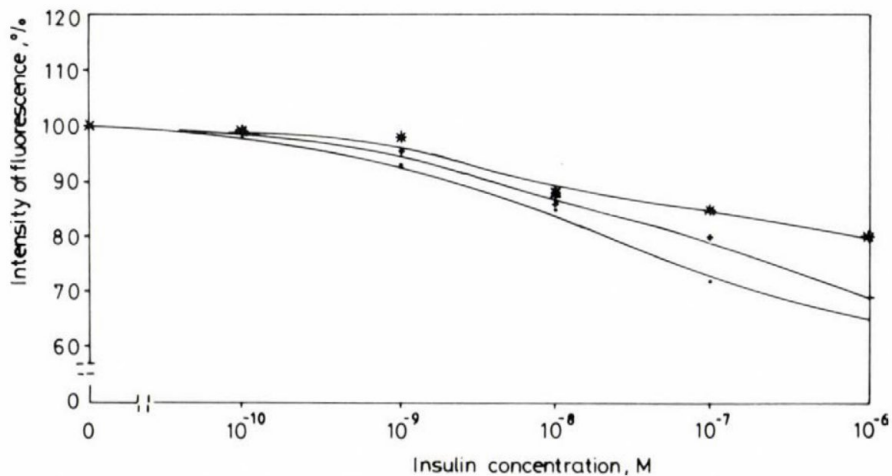


Fig. 2. Binding capacity and displacement to unlabelled insulin of insulin pretreated (imprinted) nuclei of *Tetrahymena* cells. Cells fed (-), starved for 2 h (+) and starved for 18 h (*)

This means that the effect of the one hour insulin treatment is detectable at the nuclear envelope level. Considering that the degree of displacement is an evidence of specificity, by the influence of imprinting the binding capacity of nuclear envelope turned into more specific.

The 2 h starvation of imprinted *Tetrahymena* cells suppressed the significant difference of the control to the 10^{-6} – 10^{-7} M range. This means that only a higher quantity of insulin is able to compete significantly. This shows that the number of specific receptors compared to the non specific ones is less. In the case of *Tetrahymena* starving for 18 h in a salt solution in the insulin imprinted group the curve runs more close to the control, but the difference is significant at 10^{-6} and 10^{-7} M concentrations. The binding capacity of nuclei of insulin imprinted and insulin imprinted and starved cells differs in 15% if there is a displacement with 10^{-6} M unlabelled insulin. This is identical to the difference which was detected previously [18] in one day starved and imprinted *Tetrahymena* cells on the plasma membrane. This means that at starvation the nuclear envelope and the plasma membrane have similar binding characteristics.

Considering the experiments done with cells of higher level organisms it was supposed that the insulin receptors of the nuclear envelope are structures similar or identical to the insulin receptors of the plasma membrane. It is probable that the insulin receptors of the cell surface following the binding of hormone are internalized and their lysosomal degradation is only minimal [8–10]. The rest of the receptors are transported into the nuclear envelope, and they can even get into the nucleus. According to our experiments this is possible in *Tetrahymena*, too. It is shown by the results since the nuclear envelope of imprinted cells reacted to starvation in a very similar percentual degree as the plasma membrane in our previous study [18]. On the base of the present experiments it seems to be possible that the translocation of receptors to the nucleus has an importance in the development of imprinting.

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THE VALUE OF DIFFERENT SEROLOGICAL TESTS IN SEROTYPING OF *ACTINOBACILLUS* *PLEUROPNEUMONIAE* STRAINS*

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Immune sera were raised in rabbits against 12 serotypes of *Actinobacillus pleuropneumoniae* biotype 1 in order to compare serological tests for simplicity and sensitivity in serotype determination. The serological tests included the tube agglutination test with and without 2-mercaptoethanol addition, the indirect haemagglutination test with and without 2-mercaptoethanol, the immunodiffusion test and the coagglutination test. All these tests proved suitable for serotype determination with the reference strains; however, cross-reactions of varying degree were observed in all of them. Owing to its simplicity, rapidity and specificity, the coagglutination test appears to be the most suitable for primary serotyping. A more definite answer to the question could be obtained by serotyping strains isolated from field cases.

Twelve serotypes of *Actinobacillus pleuropneumoniae* biotype 1 are known at present [1]. The varying degree of antigenic relatedness existing among the serotypes [2–6] causes cross-reactions in the serological tests. The degree of cross-reactions depends on the serological test and antigen applied [9].

The aim of this study was to raise immune sera against reference strains of the 12 known serotypes, to examine the degree of cross-reactions in the easily applicable tests, and to develop the simplest possible method for serotyping.

Materials and methods

Strains. The reference strains of the 12 serotypes of *A. pleuropneumoniae* were kindly supplied by R. Nielsen (State Veterinary Serum Laboratory, Copenhagen, Denmark).

Antigens for the immunization of rabbits. The 16-hour culture of the strains on tryptic soy agar (TSA) containing 5% yeast extract was washed off with physiological saline. This was followed by two

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cycles of washing. From the sediment a 10% suspension was prepared and used for the inoculation of rabbits.

Antigens for the agglutination test (AgP). Six-hour mucoid colonies grown on the above culture medium were washed off with saline. The bacterial suspension was washed once and resuspended in saline containing merthiolate in 1:20000 dilution to give a suspension with an optical density of 0.4 at 550 nm.

Antigens for the 2-mercaptoethanol agglutination test (2-ME-AgP). Were as for the agglutination test, with the difference that here the density of the suspension was twice as high.

Antigens for indirect haemagglutination (IHA) and 2-ME-IHA. The bacterial cultures were washed in saline adjusted to pH 11. After resuspending, the cultures were kept at room temperature for 16 h, then centrifuged at 8000 g for 45 min, and the supernatant was used as antigen.

Antigens for agar gel precipitation (AgPP). The bacteria were washed in PBS (pH 7.4) once. Subsequently, a thick suspension was prepared and stored at room temperature for one day. The supernatant obtained after centrifugation was used as antigen.

For the coagglutination test (Co-P) the antigen was obtained as described by Mittal et al. [10].

Serological tests. *AgP:* as described by Gunnarsson et al. [11]. *2-ME-AgP:* as described by Mittal et al. [12]. *IHA:* as described by Mittal et al. [13]. *2-ME-IHA:* as for the IHA, with the difference that the PBS contained 0.1 M 2-mercaptoethanol. *AgPP:* as described by Gunnarsson et al. [14], with the difference that the diameter of the wells was 4 mm and the distance between them 6 mm. *Co-P:* as described by Mittal et al. [10].

Immunization of rabbits. This was done as described by Gunnarsson et al. [14].

Results

The results of serological tests performed with the immune sera raised in rabbits and the antigens produced from the reference strains are presented in Tables I–VI.

Table I

Cross-reactions among the 12 serotypes of A. pleuropneumoniae in the agglutination test with immune sera raised in rabbits

Antigen	Rabbit immune serum											
	1	2	3	4	5	6	7	8	9	10	11	12
1	1600	25	50	25	25	25	–	25	50	–	100	–
2	50	3200	25	–	–	25	–	50	–	25	25	–
3	25	25	1600	–	25	200	–	200	100	100	100	–
4	50	50	25	1600	–	50	200	25	25	100	100	–
5	50	25	–	–	1600	50	25	–	25	50	50	25
6	25	–	100	–	–	800	–	200	25	50	50	–
7	–	25	–	100	25	–	800	–	25	25	–	–
8	–	25	200	–	25	400	–	3200	–	50	25	–
9	100	25	25	–	–	50	–	–	1600	50	200	–
10	25	50	–	–	25	–	25	25	50	800	100	–
11	200	50	–	–	25	100	–	25	400	100	1600	–
12	–	–	–	–	25	–	–	–	25	–	–	800

Table II

Cross-reactions among the 12 serotypes of A. pleuropneumoniae in the 2-ME-Ag test with immune sera raised in rabbits

Antigen	Rabbit immune serum											
	1	2	3	4	5	6	7	8	9	10	11	12
1	1280	—	10	—	—	20	—	—	20	—	80	—
2	10	1280	—	—	—	—	—	—	—	—	10	—
3	—	—	640	—	—	40	—	40	—	—	10	—
4	—	—	—	640	—	10	40	—	—	10	—	—
5	10	—	—	—	640	10	—	—	—	—	—	—
6	—	—	20	—	—	640	—	40	10	—	—	—
7	—	—	—	40	10	—	640	—	—	—	—	—
8	—	—	80	—	20	80	—	2560	—	—	—	—
9	40	20	—	—	—	—	—	—	1280	—	80	—
10	—	—	—	—	—	—	—	—	—	640	20	—
11	40	20	—	—	10	20	—	—	80	10	1280	—
12	—	—	—	—	—	—	—	—	10	—	—	320

Table III

Cross-reactions among the 12 serotypes of A. pleuropneumoniae in the indirect haemagglutination test with immune sera raised in rabbits

Antigen	Rabbit immune serum											
	1	2	3	4	5	6	7	8	9	10	11	12
1	640	—	—	—	—	—	—	—	32	8	16	—
2	—	5120	16	16	8	16	—	32	—	8	8	—
3	—	8	1280	—	—	64	—	128	16	—	—	—
4	—	8	—	640	—	8	64	—	—	16	8	—
5	—	—	32	—	1280	16	—	8	—	8	—	—
6	—	—	32	—	8	640	—	64	—	—	—	—
7	—	16	8	64	—	—	640	—	—	—	—	—
8	—	8	64	—	—	64	—	1280	—	—	16	—
9	16	—	—	—	—	—	—	—	640	8	32	—
10	8	—	16	—	—	—	—	—	—	1280	16	—
11	32	—	—	—	—	—	—	—	32	8	320	—
12	—	—	—	—	—	8	—	—	—	—	—	640

Data in Table I show that in the agglutination test minor cross-reactions occurred in all relations, though with immune sera raised against serotypes 4 and 12 these were negligible. The homologous titres were consistently by at least four dilution

steps higher than the highest heterologous values. As compared to results obtained in the agglutination test, in 2-ME-AgP the homologous titres decreased at a variable rate (Table II). The heterologous titres also underwent a similar decrease, and the majority of the lower titres disappeared.

The titres obtained for the immune sera varied within wide ranges (1:320–1:5120) in the IHA (Table III). Cross-reactions occurred in fairly large numbers in the IHA as well; however, titres exceeding 1:16 were found only for related serotypes (serotypes 3, 6 and 8; serotypes 1, 9 and 11; serotypes 4 and 7), with the exception of the titre obtained for antigen no. 2 with serum no. 8. Also in this test, the homologous titres exceeded the heterologous ones by several dilution steps. Using the IHA complemented with 2-mercaptoethanol, the homologous titres decreased moderately while the heterologous ones showed a more expressed decrease and the majority of them even disappeared (Table IV).

In AgPP (Table V) and Co-P (Table VI) slight cross-reactions were observed mainly for the related serotypes, but for these serotypes they were consistently demonstrable.

Table IV

Cross-reactions among the 12 serotypes of A. pleuropneumoniae in the 2-ME-IHA test with immune sera raised in rabbits

Antigen	Rabbit immune serum											
	1	2	3	4	5	6	7	8	9	10	11	12
1	640	–	–	–	–	–	–	–	10	–	10	–
2	–	1280	–	–	–	–	–	20	–	–	–	–
3	–	–	640	–	–	40	–	40	10	–	10	–
4	–	–	–	640	–	–	32	–	–	10	20	–
5	–	–	–	–	1280	–	–	–	–	–	10	–
6	–	–	20	–	–	640	–	40	–	10	10	–
7	–	–	–	40	–	–	640	–	10	–	–	–
8	–	–	20	–	–	40	–	1280	–	10	–	–
9	10	–	–	–	–	10	–	–	640	10	40	–
10	–	10	–	–	–	–	–	–	–	640	–	–
11	20	–	–	–	–	–	–	–	20	–	320	–
12	–	–	–	–	–	–	–	–	–	–	–	320

Table V

Cross-reactions among the 12 serotypes of A. pleuropneumoniae in the 2-ME-IHA test with immune sera raised in rabbits

Antigen	Rabbit immune serum											
	1	2	3	4	5	6	7	8	9	10	11	12
1	+	-	-	-	-	-	-	-	±	-	±	-
2	-	+	-	-	-	-	-	-	-	-	-	-
3	-	-	+	-	-	±	-	±	-	-	-	-
4	±	-	-	+	-	-	±	-	±	±	-	-
5	-	-	-	-	+	±	-	-	-	-	-	-
6	-	-	±	-	-	+	-	±	-	-	-	-
7	-	-	-	±	-	-	+	-	-	-	-	-
8	-	-	±	-	-	±	-	+	-	±	-	-
9	±	-	-	-	-	-	-	-	+	-	±	-
10	-	-	-	±	-	-	-	-	-	+	-	±
11	-	-	-	-	-	-	-	-	±	-	+	-
12	-	-	-	-	-	-	-	-	-	±	-	+

± The number of precipitation bands was lower, or the bands were less expressed, than those obtained with the homologous antigen

Table VI

Cross-reactions among the 12 serotypes of A. pleuropneumoniae in the coagglutination test with immune sera raised in rabbits

Antigen	Rabbit immune serum											
	1	2	3	4	5	6	7	8	9	10	11	12
1	++++	-	-	-	-	-	-	-	+	-	+	-
2	-	++++	-	-	-	-	-	-	-	-	-	-
3	-	-	++++	-	-	+	-	++	++	-	-	-
4	-	-	-	++++	-	-	++	-	-	-	-	-
5	-	-	-	-	++++	-	-	-	-	-	-	-
6	-	-	-	-	+	++++	-	++	-	-	-	-
7	-	-	-	+	-	-	++++	-	-	-	-	-
8	-	-	+	-	-	++	-	++++	-	-	-	-
9	+	-	++	-	-	-	-	-	++++	-	+	-
10	-	-	-	-	-	-	-	-	+	+++	+	-
11	+	-	-	-	-	-	-	-	+	-	++++	-
12	-	-	-	-	-	-	-	-	-	-	-	+++

Discussion

For the routine serotyping of *A. pleuropneumoniae* strains, Gunnarsson et al. [14] found the most suitable the agglutination test if the rabbits were immunized with whole cells not subjected to heat treatment and if a capsular bacterium suspension was used without heat treatment. In this study, we used a similar method, also with good results. It should be remarked, however, that the above-mentioned authors found practically no cross-reactions in the agglutination test (true enough that they studied only serotypes 1–5 and strain 202), while in our study substantial cross-reactions occurred, primarily between the related serotypes (serotypes 3, 6 and 8; serotypes 1, 9 and 11; serotypes 4 and 7). Similar cross-reactions were observed in the agglutination test also by others Mittal et al. [15] whereas other authors found the agglutination test unsuitable for serotyping (Lombin et al. [16]). In the agglutination test using 2-mercaptoethanol the titres were in most cases by 1–3 dilution steps lower than in the same test performed without 2-mercaptoethanol. The homologous titres and, to a somewhat larger extent, the heterologous titres decreased, and a substantial part of the lower titres disappeared. However, the heterologous titres did not disappear completely, as opposed to what was reported by certain authors (Mittal et al. [15]).

Several authors [9, 13] consider IHA the most specific test in serotyping if a salty extract is used for sensitizing the red blood cells. Mittal et al. [13] did not find any cross-reactions among serotypes 1–9. Although Gutierrez et al. [9] observed low-intensity cross-reactions between several serotypes (the cross-reaction between serotypes 4 and 7 was pronounced), they found that test more specific than the complement fixation test and ELISA. Our results resemble those of these latter authors, as in the IHA test we observed cross-reactions between the serotypes in low titres but these cross-reactions did not markedly affect the serotypability of the strains. In IHA with 2-mercaptoethanol the majority of low-titre cross-reactions completely disappeared and the higher titres decreased.

In the agar gel precipitation test, the serotypes were clearly distinguishable by the number and intensity of the precipitation bands, though cross-reactions did occur, particularly between the related serotypes (Table V). Other authors reported similar findings [7, 16].

The coagglutination test was developed for the serotyping of *A. pleuropneumoniae* strains by Mittal et al. [10]. By this test, all strains isolated from field cases (including the polyagglutinable and autoagglutinable strains) could be serotyped and no cross-reactions were observed at all among serotypes 1–8 [15]. In harmony with these results, we also found the coagglutination test a serotype-specific, sensitive, simple, rapid and reproducible method. However, we observed minor cross-reactions (Table VI).

Gutierrez et al. [9] emphasize that the degree of cross-reactions is basically influenced by the method of antigen preparation. Although we did not perform such studies, we tried to carefully follow the methods that the above-cited authors had found the most suitable for serotyping. Cross-reactions of varying degree occurred with all methods tested; however, these did not markedly affect the serotypability of the reference strains by any of the tests. Further studies are needed to determine which tests are the most specific and simple for serotyping strains isolated from field cases.

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DETERMINATION OF SEROTYPE-SPECIFIC ANTIBODIES TO *CHLAMYDIA TRACHOMATIS* USING A MICROIMMUNOFLUORESCENCE METHOD

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Serotype-specific antibodies for *Chlamydia trachomatis* were determined with a microimmunofluorescence method. The sera were obtained from 90 primary or secondary infertile women admitted for examination and from 180 control women admitted for interruption. Positive *C. trachomatis* serotype-specific antibody titres were found in 40 of the infertile women (44.4%) and 44 of the controls (24.4%). On the basis of determination of the serotype-specific antibodies, the prevalence of I, D and G serotypes was estimated.

Sexually transmitted diseases caused by *Chlamydia trachomatis* occur with high frequency throughout the world [1–5]. Whereas the determination of species-specific IgA, IgM and IgG antibodies and the cultivation of bacteria on the McCoy cell culture is simple, the determination of serotype-specific antibodies for trachoma and lymphogranuloma venereum (LGV) biotypes is time and labour-consuming [6, 7].

Materials and methods

Serum samples from 90 primary or secondary infertile women examined at the Department of Gynaecology, Albert Szent-Györgyi Medical School, and from 180 control women admitted to the same department for interruption were investigated.

Strains. *C. trachomatis* strains stored at –80 °C or in liquid nitrogen were thawed at room temperature and stirred with glass beads, RPMI 1640 medium, containing glucose and 5% fetal calf serum was then added at equal volumes. The strains were inoculated at 0.5 ml aliquots in 3 parallels onto 24 h McCoy monolayers. The cultures were centrifuged at 3000 rpm for 1 h on the cell monolayers, then incubated at 37 °C for 2 h. After removal of the medium, fresh RPMI 1640 medium containing glucose and cycloheximide was added to the cultures. Incubation lasted for 48–72 hours at 5% pCO₂ tension at 37 °C [8]. One of the parallel cultures was fixed for 10 min in methanol after removal of the medium, then stained with FITC-labelled antichlamydia monoclonal antibody (*Chlamydia trachomatis* Culture Confirmation test, SYVA MicroTrak), which binds to the membrane protein of *C. trachomatis*, thereby permitting visualization of the elementary bodies (EBs), intermediate bodies and reticulate bodies (RBs). Round cover slips were washed in PBS (pH 7.2), rinsed in distilled

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water and then dried at room temperature. After covering with PBS-glycerol, cultures were examined at a magnification of 400 under a fluorescence microscope. If sufficient RBs and EBs were found on microscopy, the strains were titrated. If the cultivation method did not reveal a sufficient number of EBs, the strain in question was passaged in embryonated eggs in order to increase the yield.

Egg inoculation. One hundred μ l aliquots of the strains were inoculated into the yolk sac of 7-day embryonated eggs. The eggs were then incubated in a brooder at 38 °C for 7 days. Before the eggs were opened, the embryos were controlled: only the yolk material of living embryos was examined. The content of the eggs was transferred to Petri dishes. The yolk sacs were cut open and emptied, then separated into two parts in sterile centrifuge tubes and after adding SP-2 medium they were at -20 °C. A small sample from a site near the umbilical vessels in each yolk sac was cut out, and dropped on a slide, and a thin smear was then made. After fixing and staining (FITC-labelled antichlamydia monoclonal antibody, Orion Diagnostica, Espoo, Finland) the smears were checked under a fluorescence microscope. If there were enough EBs in the smears, they were passed on McCoy cell culture, and the strain was then titrated.

The yolk sac membrane of the embryonated eggs was stirred strongly with sterile glass beads in 10 ml SP-2 medium. After centrifugation, a dilution series was made from the supernatant in SP-2 medium: 1:50, 1:75, 1:150 ..., up to 1:4800. A 0.5 ml portion of the diluted samples was placed on McCoy cover slips for 24 h, and the samples were then cultivated and checked in the above-mentioned way.

Titration of *C. trachomatis* antigens. Undiluted samples and 1:2, 1:4, 1:6 and 1:8 dilutions in SP-2 medium of the supernatant of the yolk sac material were dropped with the help of a pen tip onto slides previously cleaned with alcohol. The antigen dots were stained with *C. trachomatis* specific FITC-monoclonal antibodies produced by Orion Diagnostica. The highest dilution at which the brightly stained EBs were visible under the microscope was subsequently used to prepare pools.

Assembling of TRIC pools. Pool A: strains TRIC A, B, C; Pool B: strains TRIC D-K; Pool C: strains LGV I-III; Pool D: negative control (uninoculated yolk sac suspension). The pools were prepared so that the selected dilutions of the individual strains were mixed at equal portions. After drying at room temperature (minimum 2 h), the slides were fixed in -20 °C acetone, and then stored at -20 °C until use.

Serum titration. The serum samples were diluted 1:16-1:256 in PBS (pH 7.2), and the dilutions were dropped onto microimmunofluorescence (MIF) slides. The serum dilutions were incubated for 1 h in a moist chamber at 37 °C, then washed 3 times in PBS (pH 7.2), rinsed in distilled water and dried at room temperature. In previously titrated dilutions of TRIC pools of FITC-antihuman IgM and IgG conjugates were dropped onto the slides. After washing, rinsing and drying, the samples were covered with PBS-glycerinol and evaluated under a fluorescence microscope at a magnification of 400x. Besides MIF slides containing the pools, MIF slides containing the individual (*C. trachomatis* D, E, F, G, H, I and K) antigens were prepared and the serum dilutions were dropped onto the MIF slides containing the individual antigens in the manner described above.

Results

Serum samples from 90 primary or secondary infertile women and 180 controls admitted for interruption were examined as described above to determine serotype-specific *C. trachomatis* antibodies. Serum dilutions of 1:64 or above were regarded as positive. Positive A, B or C serotype-specific antibodies responsible for trachoma were not detected. I, D and G serotype-specific antibodies were found most frequently in the serum samples from both infertile and control women, but E, F, H and K serotype specific antibodies were also identified. Serotype-specific antibodies were demonstrated in the serum samples from 44.4% of the infertile women and 24.4% of

the controls (Table I). In three cases 2 and in one case 3 different serotype-specific antibodies were found. The effect of the LGV-II cross-reaction was observed. LGV was not diagnosed during the clinical examinations. Only the positive titres of the D-K serotype-specific antibodies were taken into consideration.

Table I

Distribution of C. trachomatis serotype-specific antibodies in serum samples from infertile and control women

		Serotypes							Total
		D	E	F	G	H	I	K	
Infertile group (n:90)	No.	7	3	3	6	5	12	4	40
	%	7.8	3.3	3.3	6.7	5.6	13.3	4.4	44.4
Controls (n:180)	No.	10	1	6	7	3	15	2	44
	%	5.5	0.6	3.3	3.9	1.7	8.3	1.1	24.4
Total (n:270)	No.	17	4	9	13	8	27	6	84
	%	6.3	1.5	3.3	4.8	3.0	10.0	2.2	31.1

Discussion

C. trachomatis serotypes may be determined by immunotyping the isolates, using polyclonal or monoclonal antibodies [9, 10]. The method applied may be MIF or ELISA. The use of monoclonal antibodies made it possible to improve the differentiation of strains by introducing newer serotypes. In 1991, Wang et al. [11] reported 3 new serotypes: Da, Ia and L IIa serotypes. In the present experiments, in the serum samples from both infertile and control women, I, D and G serotype-specific antibodies were most frequent.

In 1983, Kuo et al. (in Seattle) found a prevalence of E, F and D serotypes, and in 1988, Wagenvoort et al. (in The Netherlands) reported a prevalence of E, F, D and K serotypes, revealed by immunotyping the isolated strains [10, 12].

The indirect MIF method that we have introduced requires long and thorough preparatory work. However, serotype-specific antibodies may be determined on the MIF slides during a short time.

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EXAMINATION OF VEROCYTOTOXIN PRODUCING CAPACITY AND DETERMINATION OF THE PRESENCE OF SHIGA-LIKE TOXIN GENES IN HUMAN *ESCHERICHIA COLI* ISOLATES

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A total of 93 wild type *Escherichia coli* of human origin (mostly representing intestinal isolates from Hungary) were examined for the presence of Shiga-like-toxin (SLT) genes using SLT-I, SLT-II and enterohaemorrhagic *E. coli* (EHEC) specific DNA probes. The structural genes of the above specificity were labelled by random priming using 32-P-dCTP. *E. coli* strains investigated represented 16 serogroups: O1, O2, O4, O5, O6, O18, O25, O26, O45, O55, O111, O125, O126, O128, O157, O165, members of which were likely to produce verotoxin (VT) and strains of serotypes O11:NM, ONT:NM isolated from six haemorrhagic uraemic syndrome (HUS) patients. Out of these strains 51 were examined for in vitro VT production capacity. Only one strain (an O26:H11 from Germany) produced VT. This was also the only strain which proved to be positive with one of the SLT probes (SLT-II), and with the EHEC probe. From this strain a phage was isolated which was proven to be nonconvertible. These data support epidemiological and clinical observations about the very low occurrence or absence of EHEC in Hungary, in contrast to many European countries.

Haemorrhagic colitis (HC) has been first recognized as a unique disease entity in 1982, as a result of investigation of two independently occurring epidemics, caused by *Escherichia coli* O157:H7 [1–4]. The *E. coli* isolates of the rare serotype O157:H7 have not shown any traditional *E. coli* virulence character at that time. These isolates did not produce heat stable or heat labile enterotoxins, characteristic to enterotoxigenic *E. coli* (ETEC), failed to show the invasive characteristics of enteroinvasive *E. coli* (EIEC), and had no strong in vitro adhesive capacity described for enteropathogenic *E. coli* (EPEC). However, all wild strains of *E. coli* O157:H7 produced a cytotoxin detectable on vero cells and all carried a large plasmid. The clinical feature of *E. coli* O157 infection differed from that of other diarrhoeal diseases caused by *E. coli*. Thus,

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the common name "enterohaemorrhagic *E. coli*" (EHEC) was suggested for this group [2].

Between 1982 and 1989 Wachsmuth et al. [3] described 14 major epidemics caused by EHEC O157 in the North American Continent. Since 1992 the number of sporadic cases has also been increased [5]. Very soon, several other *E. coli* serogroups were also assigned to the EHEC group [6–9], which mainly caused sporadic cases of the disease [10–13]. The prototype and the most characteristic strain of the group belongs to serogroup O157:H7 [6]. According to Pai et al. [12] the VT producing *E. coli* (VTEC) was the most frequent human enteric pathogen in Calgary (Canada) during the late eighties, and 78% of the VTEC strains proved to be O157:H7. In 1985–86 after *Salmonella* and *Campylobacter* *E. coli* O157:H7 was the third most frequent bacterial enteric pathogen in Washington State [5], preceding *Shigella*. The most frequent source of O157:H7 infection was beef hamburger and unpasteurized milk [3, 13].

Wild type isolates of *E. coli* O157:H7 usually produce large quantities of two, antigenically distinct toxins, the genes of which reside on the chromosome and can be transmitted by converting phages [8, 14–24]. These toxins are mentioned under the names shigella-like toxins (SLT), or verotoxins (VT). The former name (SLT) emphasizes the similarities in structure and antigenicity to the toxin of *Shigella dysenteriae* 1 [17, 21, 25]. The homology between the structural genes of SLT-I and the Shiga toxin is > 99%. The homology between the structural genes of SLT-I and SLT II is 60%. The three toxins are composed of an A (active) and B (binding) subunit [17]. The name verotoxin emphasizes the fact that among several cell lines, vero cells are the most suitable for detection of the biological activity of the toxin [26]. In this report we use the name SLT-I and SLT-II.

Besides haemorrhagic colitis [2, 8, 27] *E. coli* O157:H7 may cause haemorrhagic uraemic syndrome (HUS) [11, 12], non bloody diarrhoea [3, 6, 13] and thrombotic thrombocytopenic purpura (TTP) as well [3, 6, 13]. Although the pathogenic role of SLT has been proven by several clinical [8, 11, 27] and experimental [19, 28–30] observations, the pathomechanism of the disease is still not completely understood. In developed industrial countries there is a trend for increased occurrence and predominance of EHEC among intestinal infections. In Hungary, there have not so far been reports on O157:H7 isolates or on typical cases of EHEC infection.

In this paper we attempted to estimate the significance of EHEC in Hungary, by investigating several faecal isolates of *E. coli* strains representing serogroups mostly other than O157:H7 isolated from cases of diarrhoea. For this purpose we used biological assay and genetic probes.

Materials and methods

Bacterial strains. Altogether 93 *E. coli* strains (isolated from humans) were tested. The strains represented the following 16 serogroups with the numbers of strains tested given in parenthesis: O1 (4), O2 (6), O4 (3), O5 (4), O6 (3), O11 (1), O18 (5), O25 (1), O26 (10), O45 (3), O55 (9), O111 (23), O125 (3), O126 (5), O128 (1), O157 (6), O165 (1). Further 5 strains were nontypable. Seventy-six strains used in SLT and EHEC specific DNA probes were from the collection of the Phage Department

of the National Institute of Hygiene. Serotyping of these strains was done by the Hungarian National *E. coli* Reference Laboratory, Department of Bacteriology, National Institute of Hygiene. Eleven strains of serogroup O111 were supplied by Dr. Judit Horváth, Csongrád County Institute of the National Public Health Service, Szeged. Further 6 *E. coli* strains isolated from faeces of HUS patients were provided by St. László Hospital, Budapest (out of which 5 were nontypable and one was O11). For the DNA probes, *E. coli* O157:H7 strains 7785 and 2-45 were used as positive controls [31]. Besides, strain 555 (O157:H7) isolated in the US and its plasmidless derivative [32] were also tested using the SLT-I and SLT-II specific DNA-probes. The HB101 strain served as negative control. Fifty-one wild human strains belonging to serogroups O26 (17), O55 (20), O111 (12) and O5 (2) were examined for verocytotoxicity.

Recombinant plasmids. pJPN37-19 and pNN111-19 containing the SLT-I and SLT-II specific DNA fragments, respectively, were kindly provided by Dr. J. Newland et al. [15, 16]. Isolation and analysis of plasmid DNA was performed according to Birnboim and Doly [33]. The restriction enzymes BamHI and PstI were used as described by the producer (Amersham International, UK).

Isolation of the SLT-I and SLT-II probe specific fragments was done as described [15]. Briefly, the plasmids pJPN37-19 and pNN111-19 were digested with BamHI and PstI, respectively, and run in agarose gel. The smaller of two fragments in each case was extracted from the agarose using the GeneClean kit (Bio 101 Inc., La Jolla, CA, USA), according to the manufacturer's description.

EHEC probe. Recombinant ~60 MDa plasmid pCVD419 (34, kindly provided by J. B. Kaper) was digested by Hind III and the 3.4 kb size fragment was isolated for use to detect homologous EHEC plasmid gene sequence in various *E. coli* strains. *E. coli* O157:H7 strains 933 and 7785 were used as positive controls, and strains 2-45 and HS as negative controls.

DNA labelling and colony hybridization. DNA fragments containing the toxinspecific sequences were labelled by using an Amersham multiprime DNA labelling system, as advised by the manufacturer. Hybond-N nylon hybridization membranes were placed onto colonies grown on McConkey agar. After 1 h at room temperature the filters were peeled off and placed colony-side up on blotting paper saturated with 0.5 M NaOH and 1.5 M NaCl for 1 min, followed by 1 min heating at 100 °C for further lysis. Samples were neutralized in a mixture of 1 M Tris (pH 7.2) and with 2 M NaCl. Filters were dried at room temperature. Hybridization conditions and autoradiography were as described by Baldini et al. [35].

Verocytotoxicity was tested as described by Konowalchuk et al. [26], using *E. coli* strains H320 (from A. O'Brien), and 2203 (O138 VT+, STa+, STb+), 2206 (O139, VT+) from our laboratory as VT positive controls, and *E. coli* 263 (O8:K87, K88ab:H19, LT+) as LT positive control. The *E. coli* 123 (O43:H28) and the K12 strain HB101 were used as negative controls. All VT tests were repeated with at least two different cell-free supernatants from 51 wild human isolates and from the controls, and the tests were run in 3-4 wells at each time. When cell rounding in at least 50% of the cells was observed, toxin neutralization was attempted with anti-LT serum (provided by Dr. H. W. Moon) and with anti-SLT serum (provided by Dr. R. A. Wilson) as described earlier [36].

Results

Successive steps in isolation of the DNA probe specific fragments are demonstrated in Fig. 1, giving details of the SLT-I purification as an example.

Hybridization. Using the SLT-I and SLT-II specific probes, the US strain 555 of serotype O157:H7 and its plasmid free derivative did react with both SLT-I and SLT-II DNA probes. None of the 92 (overwhelmingly non O157) *E. coli* strains reacted with the SLT-I probe. Only one strain (*E. coli* 91-90, of serotype O26:H11) reacted with the SLT-II probe (Fig. 2, panel B, position 4). Strain 91-90 reacted with EHEC probe, too (panel C, position 4). This strain was isolated in the former East-Germany.

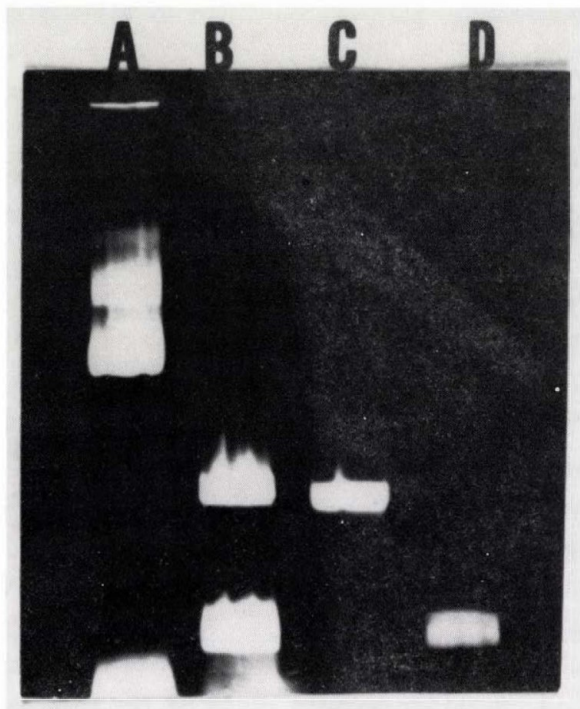


Fig. 1. Isolation of SLT-I specific DNA fragment. Samples: undigested recombinant plasmid JPN37-19(A), same plasmid digested with BamHI (B) purified cloning vector pUC19(C), and the SLT-I specific fragment of 1,142 kb (D)

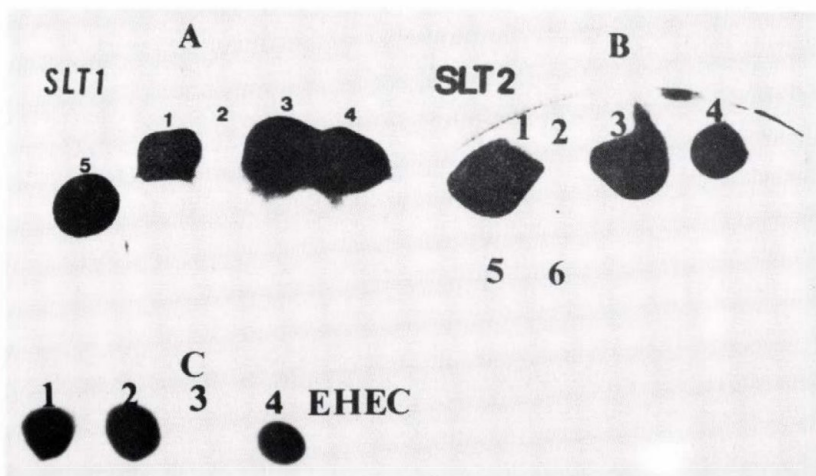


Fig. 2. In situ colony hybridization using SLT-I (A), SLT-II (B) and EHEC (C) specific probes. Panel A: *E. coli* strains 7785 (1), HB101 (2), 2-45 (3), 555 (4) and its plasmidless derivative, designated 6-14 (5) Panel B: *E. coli* strains 7785 (1), HB101 (2), 2-45 (3), 91-90 of serotype O26:H11 (4), lysogenized HB101 (5), and phage originated from strain 91-90 (6). Panel C: *E. coli* strains 933 (1), 7785 (2), HB101 (3) and 91-90 (4)

Verocytotoxicity and lysogeny. As a result of repeated blind studies, only one of the 51 strains showed marked cytotoxicity, and this was the same *E. coli* 91-90 (O26:H11) which also gave positive signal in the DNA hybridization using an SLT-II and EHEC probes. The cytotoxic effect of this strain was neutralized, by the anti-SLT serum (containing antibodies against both SLT-I and SLT-II). This strain also proved to be lysogenic. The phage was isolated and used to lyse the strain HB101 in order to test its toxin converting character, by the SLT-II DNA probe. The phage was not a converting phage: the lysogenized HB101 gave no positive signal and neither did the phage itself (Fig. 2, panel B, positions 5 and 6, respectively).

Discussion

Detection of Shiga-like toxin producing *E. coli* is one of the most significant tasks in the diagnosis of enteric infections. This is partly due to the constant increase of diseases caused by SLT producing *E. coli* in developed countries [5, 13]. In some areas such *E. coli* strains produce diarrhoea more often than *Salmonella* or *Campylobacter* [12]. The other aspect of significance is given by the severe clinical diseases: haemorrhagic colitis, haemolytic-uraemic syndrome and thrombocytopenic purpura [6, 13]. According to our best knowledge, no disease caused by *E. coli* O157:H7 has been diagnosed so far in Hungary, and the SLT producing *E. coli* is probably also of low prevalence in this country.

The diagnosis of SLT producing *E. coli* is based on the detection of Shigella-like toxin. This can be done by biological and immunological (ELISA) methods [37, 38]. Direct detection of the biological effect of the toxin is best done on vero cells [26]. However, the sensitivity and specificity of the DNA hybridization probes is far greater than those of the immunological or biological methods [15]. In our case there was a complete agreement between the results of biological and DNA hybridization tests.

In conclusion, we have adopted the SLT-I, SLT-II and EHEC specific DNA probes in our laboratory. Among 93 human isolates only one strain (O26:H11) proved to be SLT-II producer and none of the strains produced SLT-I. This O26:H11 strain was also found to be lysogenic but its phage was a non-converting one for SLT-II. These studies indicate that SLT toxins are not significant in Hungary yet, although the strains investigated represented serogroups that (based on literary data) were likely to produce such toxins. In view of the fact that the SLT toxins are encoded by prophage genes [8, 18, 19] the loss of toxicity during storage of the strains is also possible. Since our results are in contradiction with the results obtained by SLT I and SLT II producing strains in the USA and in Germany we intend to continue our examinations in a larger scale.

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BLUE-GREEN PIGMENTED SYMBIONTS OF ECHIURIDS FROM POLAR MARINE ENVIRONMENTS*

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Burrowing echiurid worms from marine sediments including sulfidic habitats of both the Antarctic Ocean and the Norwegian Sea were partly colonized by blue-green prokaryotic symbionts. These resembled free-living Prochlorophytes and contained 2-vinylpheophorbide-a5 as the only pigment being detectable by HPLC. The potential biogeochemical significance of these epizoic symbionts is discussed.

Members of the order *Echiurinea* are important mediators in the energy flow between the sea floor and the overlaying water column. They can be found in the bottom layers of box core samples as well as in dredge hauls. From the coastal shelf to the deep sea these macro-invertebrates inhabit large burrows of 3 cm and wider in diameter. A slime layer covering their body surface enables them to carry an epizoic biofilm of microorganisms from above the sediment surface to deep subsurface layers. Hence, they can be considered as vectors and promoters of microorganisms with the capacity to influence biogeochemical processes.

This is part of a phenomenon known as bioturbation [1]. In this case the gradient structure and succession of microbial and biogeochemical processes in sediments is disrupted by infaunal reworking of the sediment. Echiurids are among the largest bioturbators whose burrowing activity can reach down to sediment depths of 50 cm and below (Reichardt, unpublished).

In sediments of the Norwegian Sea (Vöring Plateau), enhanced rates of chemoautotrophic CO₂-fixation by ammonia oxidizing nitrifying bacteria were confined to those microsites in sediment profiles that represented the burrows of Echiurids [2]. This example illustrates the general importance of Echiurids for microbe-mediated biogeochemical processes in marine sediments. Another, as yet unresolved aspect, concerns the colonization of polar marine Echiurids by blue-green pigmented epibionts whose taxonomy and function are still unknown. The following is an attempt to summarize our current state of knowledge concerning this phenomenon.

* Based on presentation at the "Second International Colloquium on Microbiology in Poikilotherms" organized by the Hungarian Academy of Sciences and L. Eötvös University, Budapest, June 15–17, 1992

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Materials and methods

Echiurid specimens were obtained from Antarctic Ocean and Norwegian Sea sediments using a 50×50×60 cm – Reineck box core sampler aboard R/V “Polarstern” and R/V “Poseidon”.

For transmission electron microscopy (TEM), the pigmented slime layer was scraped off fresh collected specimens and fixed in either 4% of formaldehyde or 3% of glutaraldehyde in cacodylate buffer, postfixed in 2% of OsO₄ and embedded in Spurr’s resin following dehydration [3]. Ultra-thin sections were examined using a Zeiss EM 9 electron microscope [4].

Absorption spectra of the blue-green pigments were obtained from suspended scrapings in vivo and in methanol extracts. Chloroform extracts passed through a C18 Sep-Pak cartridge (Waters) were used for HPLC analysis using a Waters HPLC system (J. Olie, unpublished).

Assays for the assimilation of ¹⁴CO₂ and ¹⁴C-acetate were performed aboard the ship in triplicate using 1 ml aliquots of a suspension of scraped-off pigmented material in artificial seawater. These suspensions were incubated at 0 °C for 5 h in the light or the dark with 250 µl of ¹⁴C-acetate or ¹⁴C-bicarbonate. The incubation was terminated with 0.25 ml of formaldehyde and 0.1 ml of acetic acid. 10 ml of Lumagel® were added for scintillation counting [5].

Results and discussion

Incidence of blue-green Epibionts. Echiurids with blue-green pigmented epibionts were found in both Arctic and Antarctic sediments at water depths from 400 – to 1250 m (Fig. 1). Four out of ten specimens of an Echiurid tentatively classified as *Thalassema antarcticum* that had been obtained from the Antarctic Ocean were characterized by blue-green pigments. These covered their entire body surface similar to the pigmentation of *Bonellia viridis* which is common in shallow euphotic habitats. The Antarctic samples were obtained on two subsequent cruises on R/V “Polarstern” (ANT II-3 and ANT III-2) from sampling stations in Admiralty Bay near King George Island (62° 46.90 S, 57° 45.40 W and 62° 9.35 S, 58° 24.1 W, Fig. 1) in 1983 and 1984. Blue-green pigmented Echiurids were detected in both box core hauls containing sulfidic subsurface sediment layers down to approximately 50 cm depth, and dredge hauls from the sediment surface at water depths of 484 to 492 m. On a R/V “Poseidon” cruise in 1985, “pigmented” Echiurids belonging presumably to the same species were also found in box core samples of oxidized deep-sea sediment of the Norwegian Sea (67° 43.91 N, 5° 54.99 E) at 1221 m water depth.

Considering the aphotic marine habitats where “pigmented” Echiurids had been found, the occurrence of possibly photosynthetic pigments raised unanswered questions. These pigments showed red, chlorophyll-like fluorescence under UV light and were localized in coccoid epibiotic cells. Preliminary examination of fresh samples under a light microscope revealed that colonial aggregates consisting of approximately 40–60 0.9–1.2 µm large coccoid units were embedded in the epidermal mucus layer.

Ultrastructure. Later, transmission electron microscopy of thin sections of the mucus-rich material scraped off the body surface, revealed coccoid cell types of different sizes. The smaller cells of about 1 µm diameter resembled chroococcal cyanobacteria. The tight packing of thylakoids (as chromoplasm) at the cell periphery leaving a large electron-transparent zone in the center, however, was rather reminiscent of Prochlorophytes with their symbiotic genus *Prochloron* [6]. The same applied to the

presence of free thylakoids as pairs in samples where degradation of the cells had obviously reached a more advanced state.

Further TEM thin sections containing epidermal tissue showed that the coccoid pigmented cells were located as extracellular symbionts between the epidermal cells, although in a few instances the possibility of an intracellular occurrence could not entirely be ruled out. Extracellular symbiosis characterizes also the association of the prochlorophyte genus *Prochloron* with didemnic Ascidians. But these occur exclusively in tropical waters. Furthermore *Prochloron* cells are up to 20 times larger than the cells found associated with the Echiurids. On the other hand, recently free-living relatives *Prochloron* were discovered which are smaller than one μm [7]. It is therefore possible that also *Prochlorales* of smaller size exist as facultative symbionts.

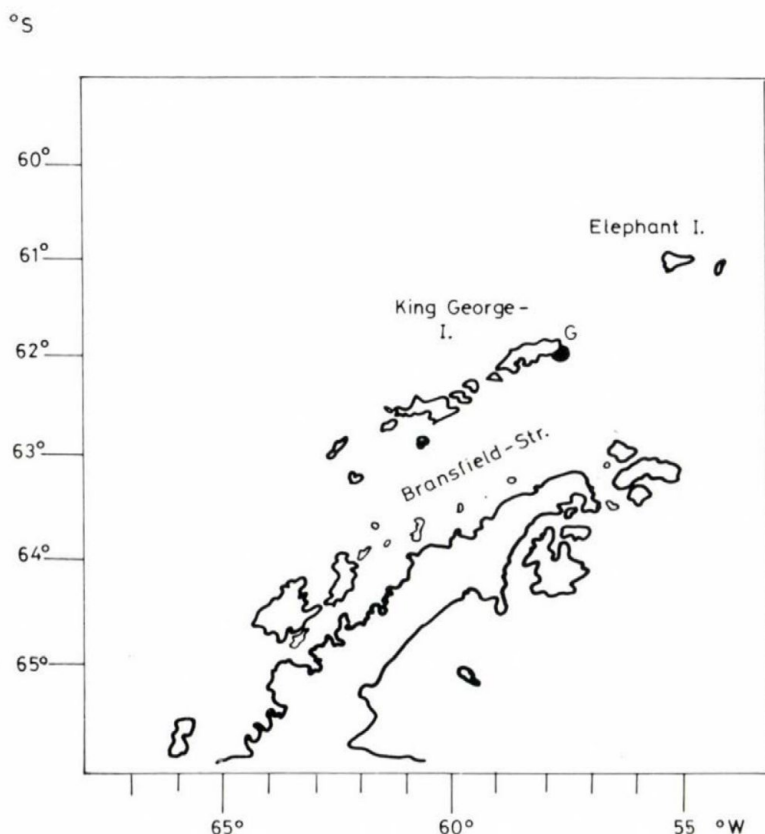


Fig. 1. Antarctic Ocean sampling station "G" in Admiralty Bay where Echiurids were found in aphotic, sulfidic sediment

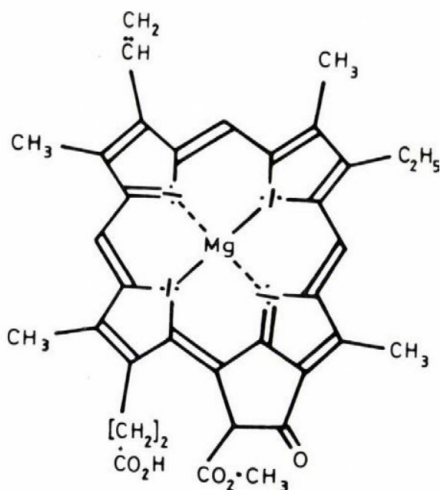


Fig. 2. Structure of magnesium 2-vinylpheophorbide a5 (monomethyl ester)

Pigment analysis. An earlier report coming rather close to suggest Prochlorophytes as symbionts of an Echiurid is an electron microscopic analysis of blue-green cells of different sizes associated with the epidermis of *Ikedosoma gogoshimense*. This Echiurid worm occurs in shallow and euphotic coastal waters and appears to be closely related to the Echiurid *Bonellia viridis* [9]. Its symbionts were described, before the order of the Prochlorales became established [6], and were classified as blue-green algae. In fact, these symbionts share certain morphological features with the epizotic microbes associated with the Echiurids from aphotic sediments in the Norwegian Sea and the Antarctic Ocean being presented in this paper.

Since pigment analysis data are missing for the symbionts of *Ikedosoma gogoshimense*, no further taxonomical clues are available in that report. Absorption spectra of cell suspensions from the blue-green pigmented cells associated with the Antarctic Echiurids did not suggest the presence of cyanobacteria, since distinct peaks of the phycobilines (phycocyanine and phycoerythrin) were missing. Even chlorophyll a was missing, whereas an absorption maximum at 638 nm was most conspicuous.

This peak appeared also in methanolic extracts and was confirmed using HPLC. It was tentatively attributed to 2-Vinyl-pheophorbide-a5 (Fig. 2). Neither chlorophylls nor bacteriochlorophylls were detected in methanolic extracts. 2-Vinyl-pheophorbide-a5 is a precursor in the biosynthesis of both chlorophylls and bacteriochlorophylls. It remains unknown whether these symbionts of the polar Echiurids are able to synthesize any potentially photosynthetic pigments beyond that compound.

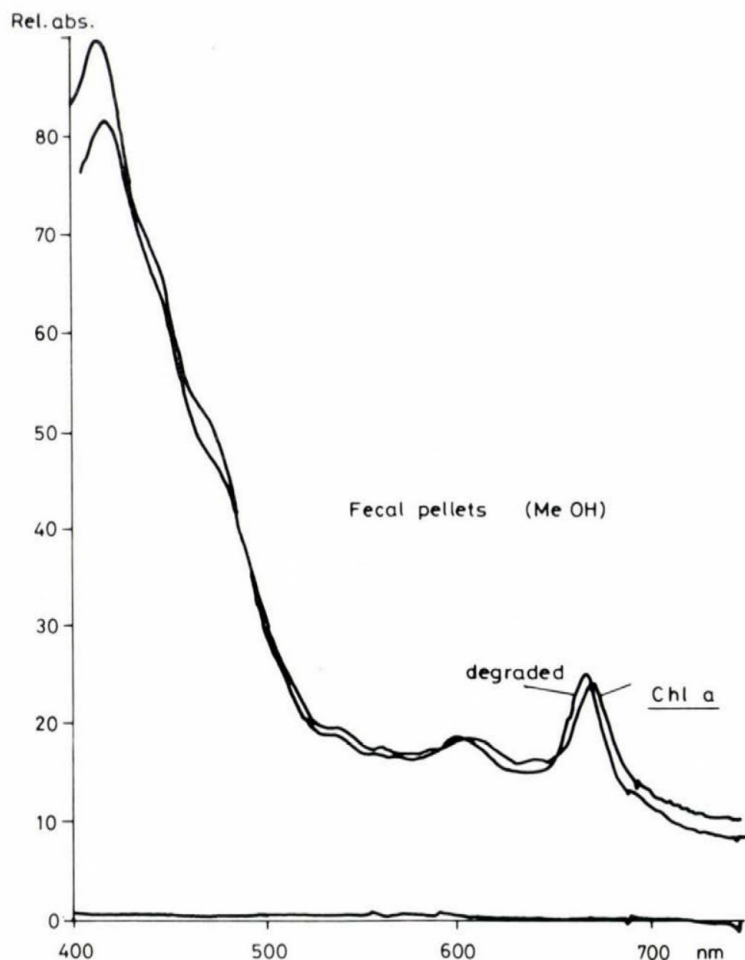


Fig. 3. Absorption spectra of methanolic extracts from Antarctic Echiurid fecal pellets, untreated (Chl a) and after heating at 100 °C (degraded), indicating ingestion of only chlorophyll a containing phototrophic organisms

Possible functions. Suspensions in sea water of fresh cells and mucus scraped off the body surface showed light-enhanced uptake of ^{14}C -labelled bicarbonate at a light intensity of $2 \mu\text{Einst m}^{-2} \text{ s}^{-1}$ for 5 h at 0 °C. Experiments carried out with these suspensions after 8 days of incubation at 0 °C, however, failed to show significant light dependent uptake of bicarbonate. Enrichment attempts on shipboard, also under anaerobic conditions, were not successful. Neither did the Echiurid worms survive until the end of the cruise; hence, further physiological experiments in the laboratory could not be carried out.

One can only speculate about the function of the blue-green pigmented symbionts in the natural and often aphotic habitat of the Echiurids. Most probably, a case of "gardening" that is defined as growth of symbiotic epizotic microorganisms being ingested as food [9], can be excluded. Methanolic extracts of fecal pellets revealed the presence of chlorophyll a, not, however, of vinylpheophorbide a5 (Fig. 3).

Table I

Photosynthetic pigments as cues to the evolution of plant chloroplasts (Chl. a, Chl. b)

CYANOBACTERIA (<i>Synechococcus</i>)	containing	Chlorophyll a + Phycobilines
PROCHLORALES (<i>Prochloron</i>)	containing	Chlorophylls a + b
ECHIUIROID SYMBIONTS	containing	2-Vinyl-pheophorbide a5

Since the described symbiotic association is only a facultative one, the symbionts are at least not permanently needed to fulfill any vital functions. As their main Antarctic habitat was sulfidic mud, it would make sense, if a functioning photosystem I were able to carry out anoxygenic photosynthesis and oxidation of sulfide. This could be a mechanism of detoxification in temporarily anoxic sulfidic sediments. Until further results come out, this case remains enigmatic.

Evolutionary aspect. There is also an evolutionistic aspect: plant chloroplasts are believed to derive from endosymbiosis involving a cyanobacterium as ancestral symbiont (Table I). Recent comparisons of certain genes of a prochlorophyte and a plant chloroplast indicate an even closer phylogenetic relationship with prochlorophytes [10]. *Cyanobacteria* possess only chlorophyll a and phycobilines, and prochlorophytes possess both chlorophylls a and b, but no phycobilines. Hence, the blue-green-pigmented symbionts of polar marine Echiurids which do neither contain phycobilines, but 2-vinyl-pheophorbide, could represent a less developed and therefore better fitting model compound for the pigments of an ancestral chloroplast.

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BACTERIA-HOST RELATIONSHIPS IN THE BIVALVE MOLLUSC *LORIPES LUCINALIS**

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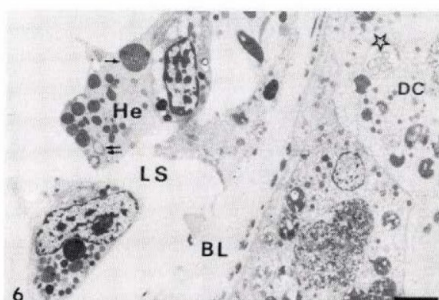
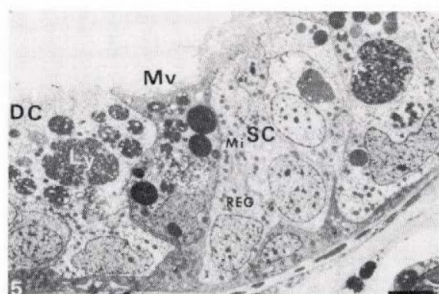
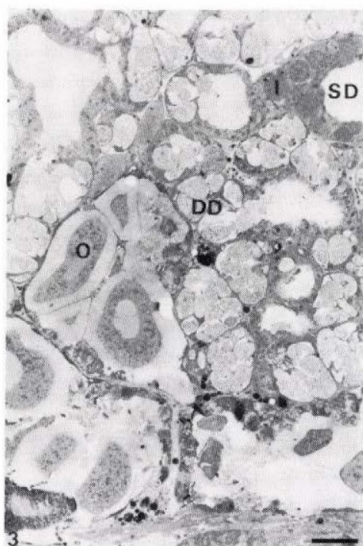
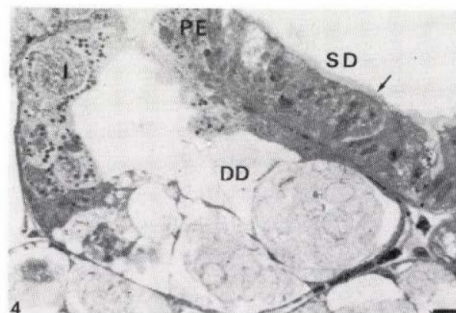
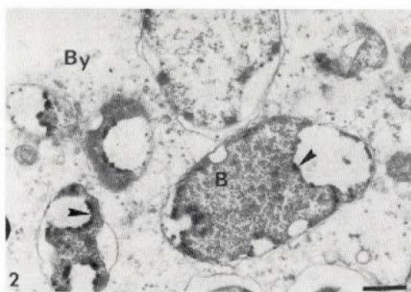
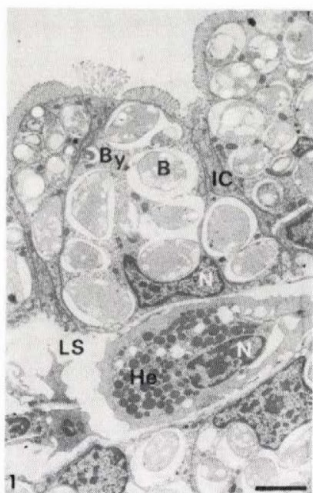
Loripes lucinalis, a lucinid species found in reduced sediments, contains endosymbiotic bacteria within specialized gill cells which contribute to the bivalve's nutrition. An additional bivalve-bacteria association can be seen in the digestive gland where large inclusion bodies filled with rickettsia- or chlamydia-like organisms are observed in the duct and tubule cells. Despite indications of a possible energy parasitism on the part of these endocellular digestive gland bacteria, the digestive epithelium of the host is not significantly damaged by the infection suggesting that this is a generalized and normal bivalve-bacteria association in adults of this species.

Since the first descriptions of a nutritional symbiosis between chemoautotrophic bacteria and mollusc bivalves in the vicinity of hydrothermal vents [1, 2], additional studies have revealed the presence of similar endocellular symbionts in bivalves from various deep-sea and littoral environments [3–11].

Loripes lucinalis, a lucinid species, can be found in certain reduced sediments of the intertidal environment and possesses, within its gills, a large number of endocellular bacteria. Studies have shown that the symbionts are sulfur oxidizing chemoautotrophs and that the carbon fixed by these microorganisms is a nutritional resource for various tissues of the bivalve host [12].

In an attempt to determine the route by which bacterial inoculation of the gill tissue occurs, the gonad tissue and in particular the oocytes were examined in the present study. It was thought that the presence of bacteria within the gonad could shed some light on the means of bacterial dispersal. Although no bacteria were found within the female gametes, microorganisms were observed in the digestive gland of *L. lucinalis*. Such an observation was possible in light of the fact that the digestive and gonadic tissues of this species are intermingled. In the present study a preliminary description of these microorganisms is given and an attempt to determine their role within the lucinid host is explored.

*Based on presentation at the "Second International Colloquium on Microbiology in Poikilotherms" organized by the Hungarian Academy of Sciences and L. Eötvös University, Budapest, June 15–17, 1992



Materials and methods

Specimens of *L. lucinalis* were collected in March, 1987 by digging at low tide (coefficient > 90) in the reduced sediment of the Moulin Blanc beach, Brest Harbour (Brittany, France). Live clams were transported to the laboratory and immediately dissected to separate gills and digestive gland. The tissues were fixed with 3% glutaraldehyde, post-fixed with 2% osmium tetroxide, and embedded in Spurr. Semi-thin sections were cut and stained with toluidine blue for light microscopy observations. For transmission electron microscopy, ultra-thin sections were cut and stained with 7% uranyl acetate and 0.1% lead citrate before examination using a 100 CX Jeol microscope.

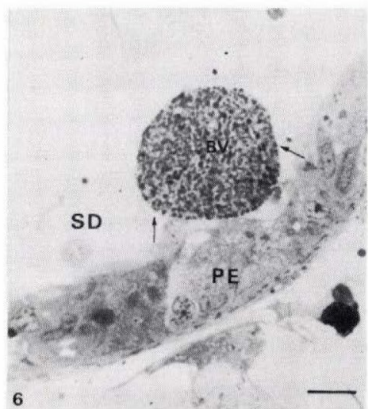
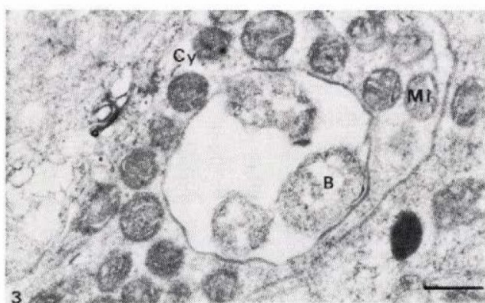
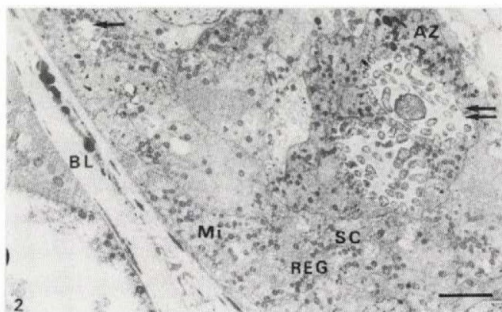
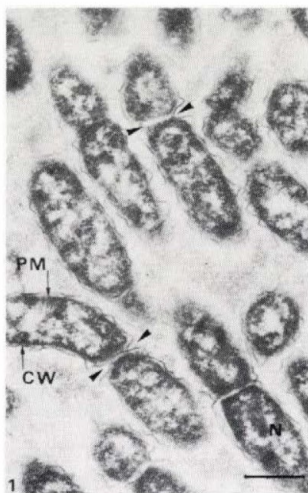
Results

Gill. The anatomy and structure of several lucinid species have already been described in detail elsewhere [12, 13]. In these studies, the gill has been described as being filled, except for a superficial area occupied by the ciliated cells, with bacteriocyte cells which contain an abundance of bacteria (Plate 1:3, 4). These bacteria have been shown to be Gram-negative sulfur oxidizing chemoautotrophs. Bacterial sizes for *L. lucinalis* range from 0.7 to 1.5×0.7 to $2.4 \mu\text{m}$. They are progressively lysed within the bacteriocyte cells (Plate 1:2).

Digestive Gland. The digestive diverticula is made up of a dendritic system of main ducts arising from the stomach of the animal. Secondary ducts, lined with a simple prismatic epithelium, give rise to the blind ending digestive tubules (Plate 1:3, 4). The digestive epithelium is made up of two cell types; the basophilic, pyramidal secretory cells and the clear, vacuolized digestive cells both of which are covered with an apical brush border (Plate 1:6).

The digestive cells are clear cylindrical cells with an irregularly shaped basal nucleus. A number of mitochondria are also found in vicinity of the nucleus. The main character of these digestive cells is the presence of a large number of vacuoles as well as secondary lysosomes and residual bodies.

←
Plate 1. (1) Electron micrograph through the gill filament showing a bacteriocyte (By), intercalary cell (IC), nucleus (N), bacteria (B), haemocyte (He), and lacunal space (LS). Scale bar = $2 \mu\text{m}$. (2) Bacteriocyte (By), showing marked bacterial lysis (arrow), bacteria (B). Scale bar = $0.5 \mu\text{m}$. (3) Light micrograph through the digestive gland showing the digestive diverticula (DD), secondary ducts (SD) with spheroidal inclusions (I), and gonad tissue with oocytes (O). Scale bar = $40 \mu\text{m}$. (4) Light micrograph through the digestive gland. Visible are the digestive diverticula (DD) with one spheroidal inclusion (I), and a secondary duct (SD) lined with a simple prismatic epithelium (PE) possessing a brush border (arrow). Scale bar = $13 \mu\text{m}$. (5) Electron micrograph through the digestive gland showing the digestive gland epithelium with two cell types covered with microvilli (Mv); the digestive cells (DC) with secondary lysosomes (Ly) and the secretory cells (SC) with many mitochondria (Mi) and an abundant rough endoplasmic reticulum (REG). Scale bar = $2 \mu\text{m}$. (6) Electron micrograph through the digestive gland. Seen is the digestive gland epithelium resting on a basal lamella (BL), the lacunal space (LS) in which are found numerous haemocytes (He) possessing dense particles (single arrow) and putative bacteria-like organisms (double arrow). Also visible within a digestive cell (DC) is a small colony of 4 bacteria (*) near the basal plasmic membrane. Scale bar = $2 \mu\text{m}$



Digestive Gland Microorganisms. Basophilic spheroidal and ellipsoidal inclusions were observed in secretory cells, and occasionally in the digestive cells of the digestive gland (Plate 2:1). Although infection of tubule cells was observed, the majority of the inclusions were observed in duct cells. Inclusion sizes were from 15 to 30 μm in diameter and were found to contain a variable number of bacteria. For these bacteria, three different forms were discerned; reticulate rod-shaped cells, giant pleomorphic cells, and deformed electron-dense cells.

Electron microscopy reveals that this epithelium rests on a basal lamella which is fibrillar in nature and against which many haemocytes are observed, many of which contain dense particles and exhibit pseudopodial extensions (Plate 1:6).

The secretory cells are characterized by a cytoplasm very rich in mitochondria and generally lack vacuoles. A spherical basal nucleus can be observed and it is in this region that many cells exhibit an abundant endoplasmic reticulum and mitochondria within the ergastoplasmic lamellae.

(i) *Reticulate rods* measure approximately 0.25 μm in diameter and 0.6 to 0.9 μm in length. They are bound by a plasma membrane and a cell wall which exhibits a rippled appearance. The interior of the bacteria is composed of granular electron-dense material giving the cells a mottled appearance. Peripheral electron-dense areas are believed to be ribosome layers while the more reticulate areas resemble DNA-fibrils. Numerous bacteria can be observed undergoing binary fission. These cells can be found singly within the host vacuoles or can form much larger colonies. When the colonies are small, they are found near the basal membrane (Plate 2:2). It is interesting to note that, at this stage, the bacterial cells are found close to the host vacuole membrane the outside of which is surrounded by a ring of mitochondria (Plate 2:3). The larger colonies, located at a more apical position in the cell, fill almost the entire host cell such that the nucleus is often compressed against the basal membrane.

(ii) *Giant pleomorphic cells* range from to 2×1 to 1.4 μm in size. Besides being larger and pleomorphic, these cells differ from the reticulate rods in that they possess clear areas filled with small electron dense particles which could be clumps of ribosomes or virus-like particles, possibly phage (Plate 2:4). These bacteria are only observed apically in very large colonies.



Plate 2. Electron micrographs through the digestive epithelium and the three different forms of bacteria encountered. (1) The reticulate rods with a plasmic membrane (PM), rippled cell wall (CW), DNA fibrils or nucleoid (N). Some bacteria exhibit a constricted zone (arrows) indicative of binary fission. Scale bar = 0.25 μm . (2) Small colonies of reticulate bacteria (arrow) which are generally located near the basal zone of the host cell and the larger ones (double arrow) near the apical zone (AZ). Secretory cells (SC) with rough endoplasmic reticulum (REG) and mitochondria (Mi). Basal lamella (BL). Scale bar = 2 μm . (3) A small colony of three reticulate rod shaped bacteria (B) surrounded by a ring of mitochondria (Mi) in the cytoplasm (Cy) of the host cell. Scale bar = 0.25 μm . (4) Giant pleomorphic cells filled with small electron-dense particles (possibly ribosomes or virus-like particles). Constriction (arrow) of the cell wall suggesting that replication takes place by binary fission. Scale bar = 0.4 μm . (5) Electron dense cells with a central vacuole (v) and a dense material (DM) in proximity of the plasma membrane. Breakdown (arrows) of the apical plasmic membrane of the host cell and release of bacteria (B) into the digestive epithelium lumen. Among these organisms are products of lysed bacteria (*), notably polyhedral shapes (thick arrow) suggesting phage. Scale bar = 0.4 μm . (6) A light micrograph showing exocytosis (arrows) of the entire bacteria-filled vacuole (BV) into the secondary duct lumen (SD). Prismatic epithelium (PE). Scale bar = 10 μm

(iii) *Electron dense cells*. In these cells, ranging from 0.25 to 0.4×0.4 to $0.7 \mu\text{m}$ in size, a central cytoplasmic vacuole appears and there is an accumulation of electron dense material against the plasma membrane (Plate 2:5). Cytoplasm shrinkage occurs which brings about a deformation of the bacteria. Exterior to the cells can be seen some unidentifiable material and electron dense particles. These could be the virus-like particles and bacterial cellular material liberated as a result of cell lysis. With the presence of these bacterial cells is seen a breakdown of the host cell membrane with the subsequent release of the bacteria into the lumen (Plate 2:6).

Basophilic inclusion bodies can also be seen free within the digestive tract lumen indicating a breakdown of the host cell membrane and exocytosis of the entire bacteria-filled vacuole.

Discussion

Over the last decade, numerous studies have been carried out in an attempt to improve our understanding of the relationship existing between bivalve molluscs and endocellular bacteria found for the most part in the gills and digestive gland. Results indicate that these bacteria can contribute to the nutrition of the host. Such is the case in bivalves from hydrothermal vent sites [5, 8, 14–16] and from reduced littoral sediments [4, 6, 12, 18–20]. In other situations, the bacterial colonization can be pathogenic to the host either at the larval [21–24] or adult stage [25–27] or, conversely, can have no noticeable deleterious effects [28–30].

In *L. lucinalis*, histoautoradiographic studies have revealed that there is transport of bacterial endosymbiontly fixed carbon from the gills towards various host tissues, notably the mantle edge and female gonad [12]. Using stable carbon-isotope ratios, Spiro et al. [31] estimated that the endocellular gill symbionts provide the host bivalve with roughly half of its nutritional requirements when ^{13}C values are between -23.4 and -22.8 . For the littoral lucinid species, the ^{13}C values allow us to estimate the endosymbiotic carbon contribution to be 40, 54, 73, and 81% for *Lucinella divaricata*, *Lucinoma borealis*, *Thyasira flexuosa* and *L. lucinalis* respectively (Diouris, pers. com.).

The anatomy of the digestive system in the Lucinaceae was described for the first time by Allen [32] and more recently Donval et al. [13] completed an ultrastructural study of *L. divaricata* and *T. flexuosa*. These studies show that, although the digestive system in these organisms is simplified with a reduction of palps and sorting areas, the digestive epithelium (ducts and tubules) are made up of the same two cell types as are found in other bivalves. These two cell types are the secretory and digestive cells. To our knowledge, this is the first study to report the presence of endocellular bacteria in the digestive gland of a lucinidae.

Based on morphological characteristics, the microorganisms which are observed in *L. lucinalis* are rickettsiales- or chlamydiales-like organisms. Generally, rickettsia-like organisms observed in bivalves have been so designated based on their rod-shaped morphology and possibility of pleomorphism. For the chlamydia-like bacteria, identification is based on the presence of the developmental stages typical of *Chlamydia* found in higher animals. In the present study, the reticulate rods, many of

which are observed undergoing binary fission, recall the *Rickettsiales* by their general morphology and presence of the typical ripple cell wall characteristic of this order. Similar organisms have been described by a number of authors and classified as being rickettsia-like [28, 30, 33–36]. Giant, pleomorphic forms have also been described in previous studies [37, 38]. The presence of these larger forms of the bacteria is open to several interpretations. They may be a second developmental stage in the infectious life cycle of the organism which resembles more a chlamydia-like organism. However, Buchanan [38] described a rickettsia which developed into giant pleomorphic forms where virus-like particles could be seen in the cytoplasm. As dense particles, possibly viruses, were observed in the cytoplasm of the bacteria in the present study, the possibility of these giant pleomorphic forms being formed as a result of a viral infection cannot be excluded. Certainly more work is required to explore this possibility as the presence of such a phage could play a role in the spread and possibly the pathogenicity of the intercellular bacteria.

The third form of bacteria observed in this study, the electron dense bodies, have also been described elsewhere [37]. These authors suggest that such cells would be in a degenerative state brought about by lysis of the microorganism due to a hyperinfection. Another possibility is that these bodies represent the infectious form of the microorganism, again, recalling the order *Chlamydiales*.

Despite the difficulties in classifying these digestive gland endocellular bacteria, some generalizations can be made as to the progression of the infection. The initial infection occurs in the basal part of the cell, as vacuoles containing a single bacterium can be observed in this area. More than one bacteria can infect the same host cell resulting in multiple vacuoles within a host digestive cell. The bacteria undergo binary fission and gradually fill up the vacuole. The close proximity of the infectious agents to the vacuole membrane and, hence, to the exterior accumulation of mitochondria would suggest that the bacteria might be drawing host-produced ATP for its growth purposes. This would indicate a form of energy parasitism. As the vacuole increases in volume and bacterial numbers, it also migrates towards the apical pole. In its final stage of development, the vacuole is at its most apical position, and is completely filled with bacteria. Ultimately, the host cell bursts releasing the bacteria or, in some cases, the entire membrane bound inclusion body is released into the digestive lumen.

Over the past fifteen years, many researchers have observed in various bivalve mollusc tissues the presence of microorganisms similar to the ones described in the present study. Rickettsia- and chlamydia-like procaryotes have been observed in clams, oysters, mussels, and scallops (for review see Fries and Grant [33]). The tissues infected include the gill epithelium [28], digestive epithelium [35, 38–40] and kidney [36]. However little is known about the pathogenic impact of these infections. Most authors agree that the observed infections appear to confer little deleterious effect on their hosts [28–30, 39]. Similarly, in the present study, the digestive epithelium was not found to be significantly damaged by the infections, even in samples where lesions were quite numerous. As has already been noted [35], the normal process of digestive epithelial cell regeneration limits the effect of the bacteria by reconstituting the epithelium while at the same time facilitating the elimination of the microcolonies. This would suggest that for *L. lucinalis*, and perhaps for other bivalves as well, these rickettsia- or chlamydia-like infections are not pathogenic to their hosts; that this

association is in fact in equilibrium and should be considered as generalized and normal in adult bivalves.

There are therefore two types of bacterial associations in the bivalve *L. lucinalis* but which represent opposite metabolic bacteria-host cell relationships. The first type of association involves the colonization by sulfur-oxidizing bacteria of specialized gill cells. The bacterial metabolites and the products of bacterial cell lysis are used for the development and reproduction of *L. lucinalis*. In the second type of association, the digestive gland serves as a reservoir for large bacterial inclusions that do not seem to adversely affect the bivalve. *L. lucinalis* provides a nutritionally favourable environment for the survival, growth and replication of these chlamydia- or rickettsia-like organisms which would seem to derive at least part of their energy from the mitochondria of the infected host cells.

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ULTRASTRUCTURAL ANALYSIS OF THE INTESTINAL CONTENT OF EARTHWORM *LUMBRICUS RUBELLUS* HOFFM. (ANNELIDA, LUMBRICIDAE)*

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Gut content sections of the earthworm *Lumbricus rubellus*, the surrounding soil and casts were studied using transmission electron microscopy. Bacteria, actinomycetes, dead fungi and empty mycelia were observed to occur throughout the gut content. In the foregut and midgut of worms, some bacteria were found to be partially or totally lysed and digested, although were protected by polysaccharides and clay particles. Not only inactive resting forms (spores) or cells protected by capsular polysaccharides and clay particles and/or by plant cellular remnants, but also separate metabolically active and dividing cells were recovered especially in hindgut. The formation of new bacterial microaggregates was noticed in the posterior intestine as well.

In contrast to conventional microbiological analyses of different soil materials, the methods of electron microscopy make possible the examination of bacteria, actinomycetes and fungi fixed and embedded in situ [1]. Methods of the transmission electron microscopy (TEM) were therefore used for the study of relations between microorganisms and soil animals, mainly of the biodegradation of organic material, ultrastructural changes and microbiology in gut contents among other earthworms [2, 3]. The most detailed analysis of gut content of the tropical earthworm *Pontoscolex corethrurus* Müller, 1857 and casts of different age was performed by Barois [4].

The aim of our research was to evaluate ultrastructural and microbial changes in the materials parallelly studied by conventional microbiological methods, i.e. by epifluorescent microscopy (Kristůfek et al., in press), the foregut, midgut, and hindgut contents of the epigeic earthworm *Lumbricus rubellus* Hoffmeister, 1843, and soil and casts from the same plot.

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Material and methods

The procedure of digging and hand sorting of soil samples was used for earthworm sampling in May 1990 and October 1991 in an allochthonous sparse wood of *Populus nigra* L. and *Alnus glutinosa* (L.) Gaertn., gleic cambisol with mull humus horizon soil type in the Stromovka Park at České Budějovice, Czech Republic. At the same time, soil samples from the upper 10 cm and surface casts (unknown age and origin) were taken. *L. rubellus*, a representative of epigeic earthworms, was used for gut content investigations. Worms were surface disinfected, dissected, and their intestines were aseptically prepared. Samples of gut contents were then taken from three sections of the intestine (foregut, midgut and hindgut) and together with soil and cast particles were processed by TEM. The material was directly prepared according to the method of Villemin and Toutain [6], i.e. the samples were fixed with 1% phosphate buffered OsO_4 for 16 h by capillarity in Millipore filter boxes with membrane filters, covered with a 2% agar droplet, dehydrated through a graded series of acetone and embedded in Durkupan resin (Polysciences). For partial dissolution of silica (quartz grains) and silicates (clay minerals), the resin blocks were treated before final sectioning with 10% hydrofluoric acid according to Saur and Ponge [6]. The ultrathin sections were contrasted by uranylacetate and lead citrate and examined using a Philips EM 420 electron microscope (80 kV).

Results

TEM analyses of the soil showed the presence of a high proportion of differently degraded plant material and the amount of microorganisms. Mainly bacteria were very active and attacked fragments of plant cells (Plate 1) and humified organic matter, i.e. brown pigments (opaque in the electron field) (Plate 1, 2). Living actinomycete hyphae (Plate 1, 3) and different metabolically active bacteria populations (Plate 1, 4) were observed in other parts of soil matter under study, as well. Soil fungi were not numerous.

Non-disturbed plant fragments with well-preserved cell structure, as well as a mixture of soil amorphous organic matter with markedly transformed plant cell walls, mineral particles and microorganisms were noted as the food of *L. rubellus*. In the gut that material was mixed with mucus substances and therefore diluted.

In foregut and midgut some bacteria were found to be lysed and digested (Plate 2, 5) even if they were protected by polysaccharides and/or clay particles (Plate 2, 6 and 8). Only empty dead fungal hyphae (Plate 2, 7) and the biodegradation of plant fragments by worm enzymatic activity (Plate 2, 9) were observed here.

Nevertheless, bacteria and actinomycetes were noted in all parts of the digestive tube of earthworms and their presence apparently increased in the hindgut. They occurred singly in gut material (Plate 3, 10) and/or were protected by plant and fungal remnants (Plate 3, 11 and 12). Spores (Plate 3, 13) and well-preserved bacterial microaggregates (Plate 3, 14) represented further forms withstanding the passage through the gut.

Separate metabolically active bacteria (Plate 4, 15), the cells of which were successively enveloped by own exopolysaccharide matrix and fine clay particles (Plate 16), including bacteria well-surrounded by two entire polysaccharide and clay strata (Plate 4, 17) confirmed the formation of new bacterial microaggregates in the hindgut of the earthworm *L. rubellus*. Extremely large microaggregates with proliferation of bacteria (Plate 4, 18) characterized the ultrastructure of the observed casts.

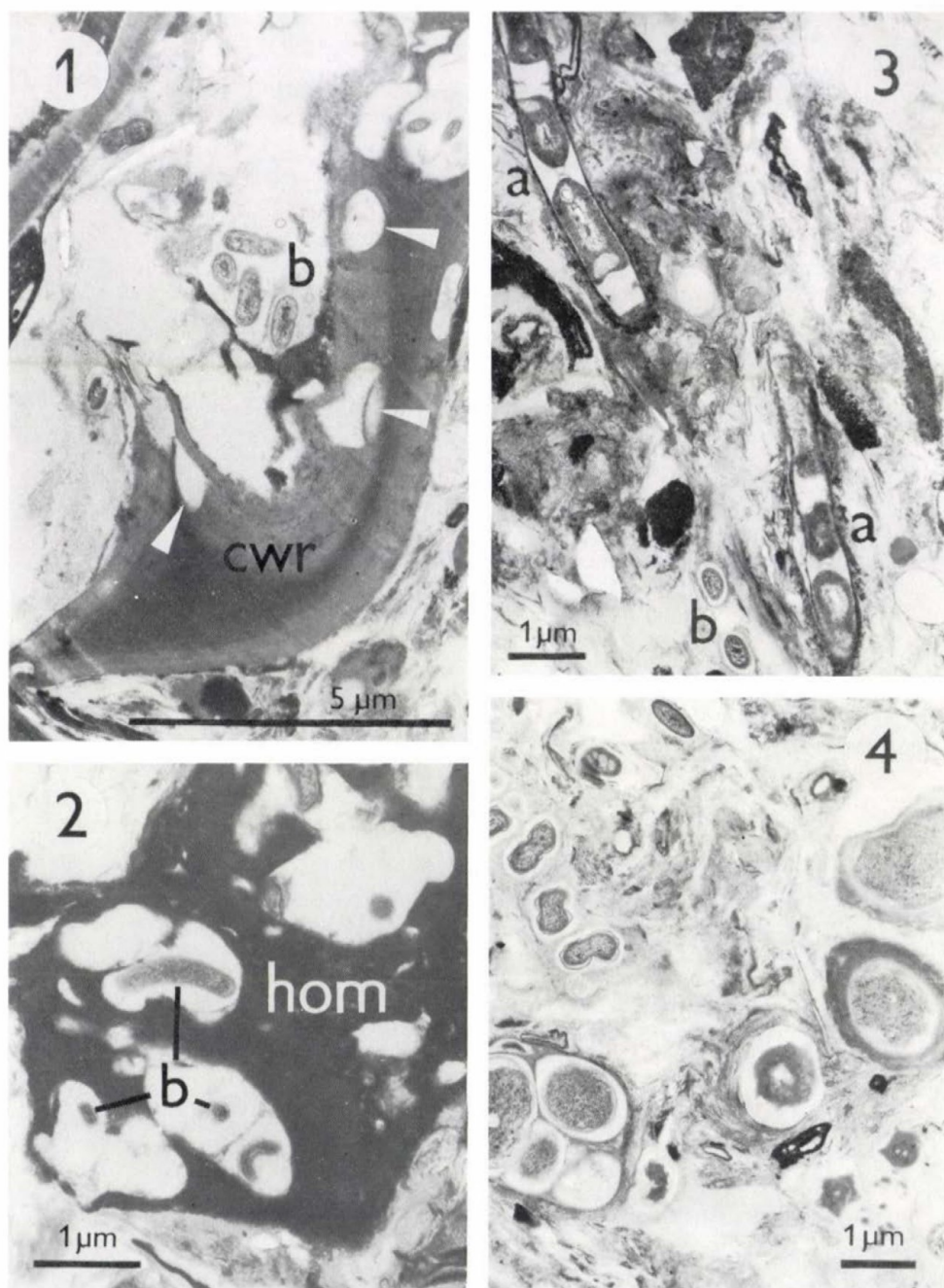


Plate 1. (1) Soil: cell-wall remnants partly lysed by microorganisms (arrows). (2) Soil: lysis of humified organic matter by bacteria. (3) Soil: actinomycete hyphae and bacteria in dense organo-mineral matter. (4) Soil: different types of bacteria in organo-mineral matter. Abbreviations: b - bacteria, cwr - cell-wall remnants, hom - humified organic matter, a - actinomycetes, c - clay minerals, h - fungal hyphae, p - polysaccharides

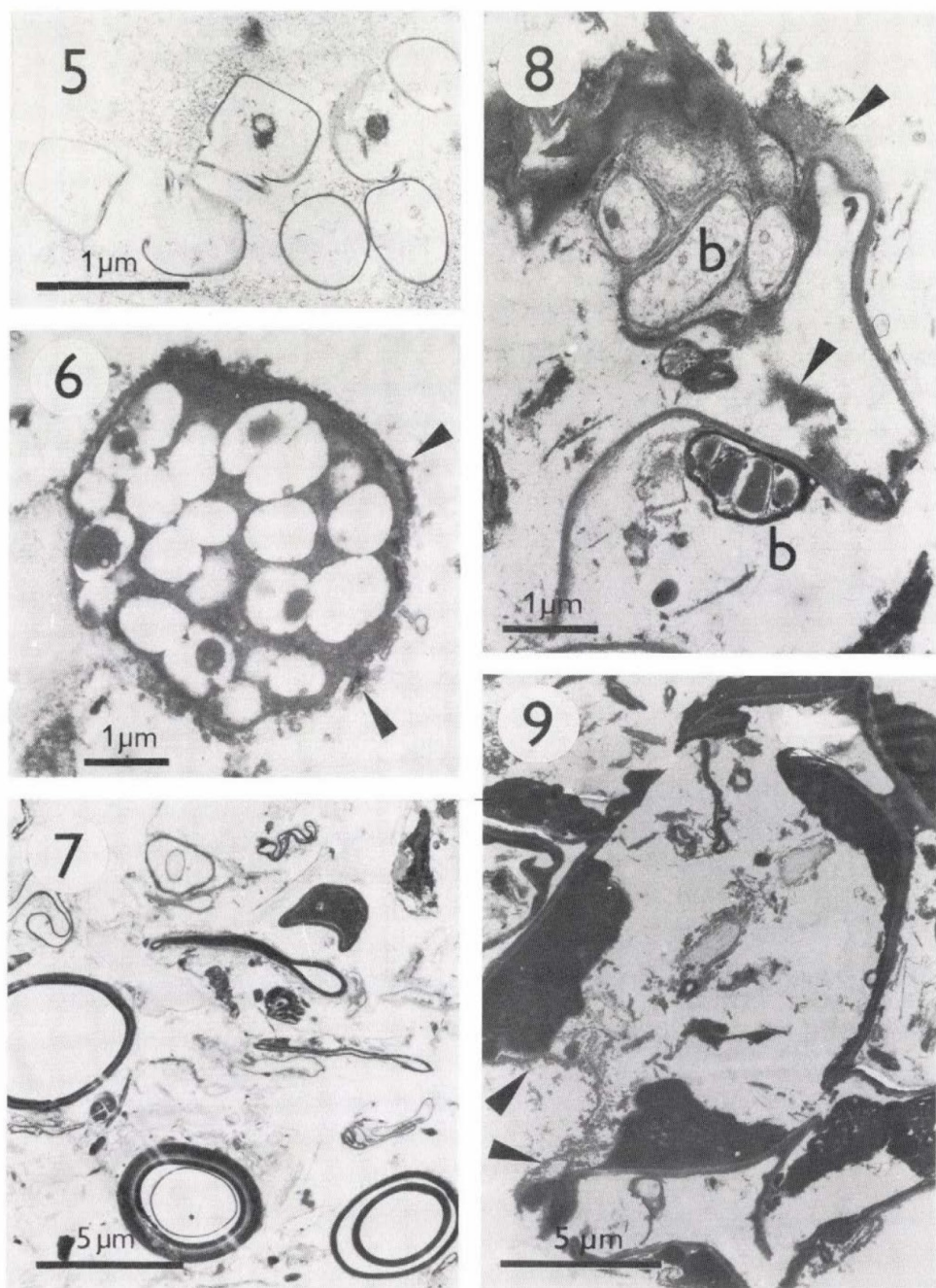


Plate 2. (5) Lysed and digested bacteria (cell-wall ghosts) in midgut. (6) Lysed colony of bacteria - microaggregate in foregut with granular enzymatic biodegradation of polysaccharides (arrows). (7) Empty dead fungal hyphae in the midgut content. (8) Lysis of bacterial colonies - microaggregates and biodegradation of plant cell remnants (arrows) in foregut. (9) Destruction and granular biodegradation (arrows) of plant cell fragments in foregut. For abbreviations see Plate 1

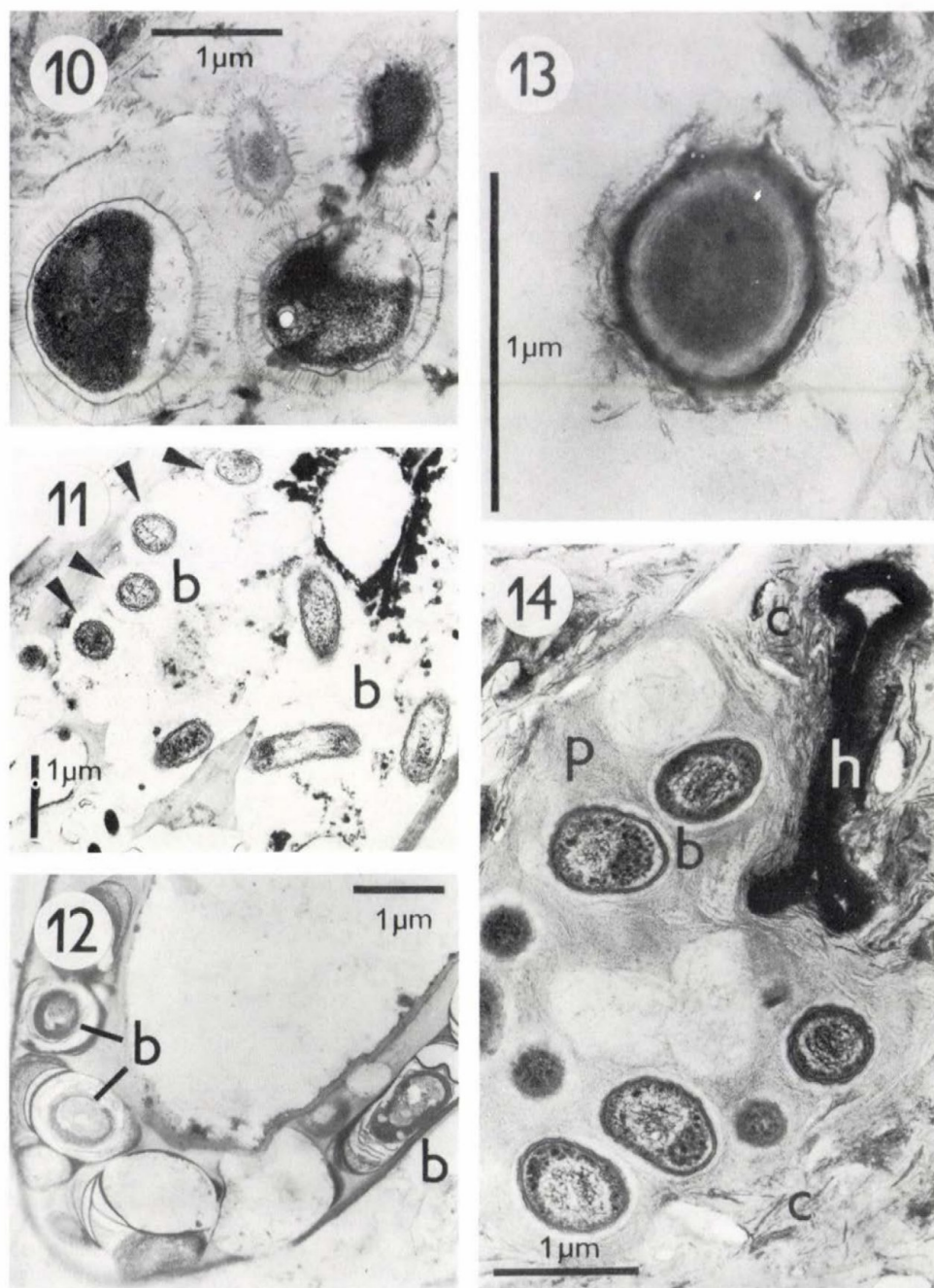


Plate 3. (10) Single bacteria in the hindgut content. (11) Activity of bacteria into degraded plant cells in hindgut content. Arrows: zones of cell-wall lysis. (12) Active bacteria in the wall of dead fungal hyphae in hindgut. (13) Bacterial spore with several fine clay minerals. (14) Bacterial microaggregate in hindgut. For abbreviations see Plate 1

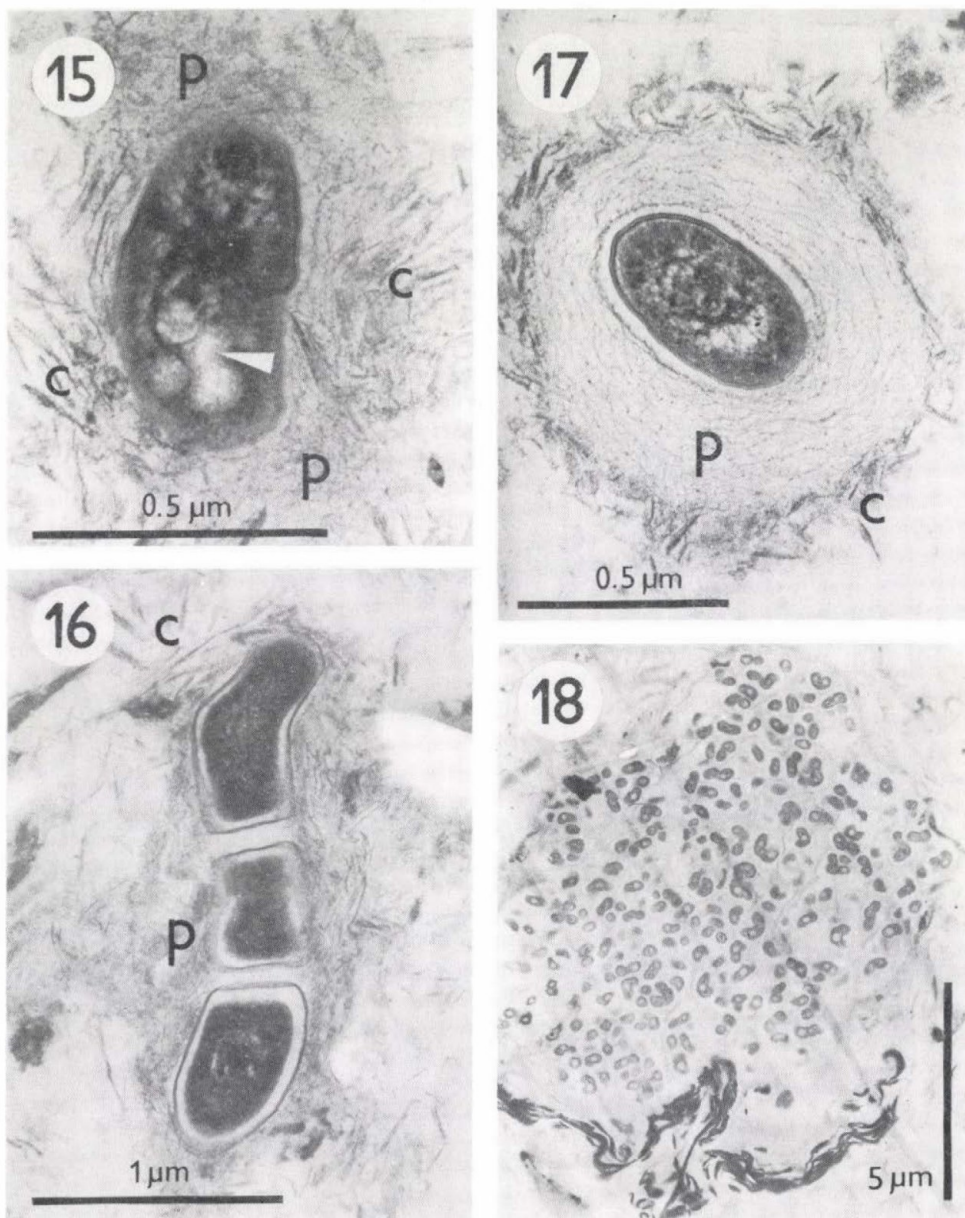


Plate 4. (15) Active bacterium in the hindgut with reserve material (arrow) and fine clay particles adsorbed directly into its cell-wall. (16) Actinomycete cells in the hindgut with own polysaccharides and clay minerals. (17) New bacterial microaggregate in hindgut with well developed polysaccharide capsule and stratum of adsorbed clay minerals. (18) Large microaggregate in cast. The active bacteria contain many reserve materials, probably polyhydroxybutyrates (light spots in cells). For abbreviations see Plate 1

Discussion

L. rubellus has been characterized by Bouché [7] as an epigeic species. Pearce [8], studying food preferences, concluded that this earthworm mainly fed on relatively fresh plant material or slightly decomposed remains rich in organic substrates, i.e. it is a detritivore. In the present study, in the gut contents of dissected animals, large pieces of differently decomposed plant material, such as leaf litter, were observed and TEM confirmed a high diversity of ingested food.

Soil fungi, bacteria, and other microorganisms are significant items in the earthworm diet [9, 10], this was confirmed by TEM methods as well. Lebreton [2] observed in the gut content of the anecic earthworm *Lumbricus herculeus* (Savigny, 1826) only the digestion of extracellular polysidic products and the release of bacteria from microaggregates and in endogeic species *Aporrectodea caliginosa* (Savigny, 1826) in addition to the lysis of these bacteria. In contrast, Rafidison [3] demonstrated by means of TEM the formation of new bacterial microaggregates in the intestine of the anecic earthworm *Nicodrilus vellox* (Bouché, 1967). Likewise, Barois [4] described through the same analysis the activation of bacteria in the gut of endogeic tropical earthworms together with the formation of colonies, which in hindgut formed microaggregates due to the adsorption of clay minerals around bacterial polysaccharide capsules.

Our results demonstrated that besides termites [11], and anecic [3] and endogeic earthworms [4], the formation of bacterial microaggregates can take place in the epigeic earthworms, too.

The presence of large microaggregates in investigated casts correlates with the excellent nutritional niche which exists. The casts are therefore foci from which soil microorganisms can spread into surrounding soil [12].

In conclusion, the microbial community in the gut content of *L. rubellus* was formed mainly by bacteria, but actinomycetes were observed in all parts of the intestine, too. Results of the TEM analysis confirmed our epifluorescent microscopy determination of microbial populations [13], showing an increase of bacterial counts especially in the hindgut of the earthworms. The observed lysis of bacteria in the foregut and midgut, as well as high activity of separate cells and formation of microaggregates in the hindgut of the earthworms are in agreement with these microbial analyses.

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A COMPARATIVE STUDY FOR DETECTION OF *CHLAMYDIA TRACHOMATIS* AND *NEISSERIA* *GONORRHOEAE* WITH DNA PROBE

(A NOTE)

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A newly developed DNA probe assay (Gen-Probe Pace 2 San Diego, USA) was compared with *Chlamydia trachomatis* direct immunofluorescence and *Neisseria gonorrhoeae* culture. Detection of *C. trachomatis* in cervical specimens from women and urethral specimens from men showed 23% positivity out of 313 cases. Out of the 69 positive cases 40 were positive with both examinations, in 29 cases only with DNA probe. Examinations of *N. gonorrhoeae* in 254 patients gave 98% positivity. Sensitivity of DNA probe assay was 100%, specificity was 97.8%. On the basis of preliminary data Gen-Probe is suitable for the detection of both causative agents.

Urogenital infections caused by *Neisseria gonorrhoeae* and *Chlamydia trachomatis* belong to the most frequent sexually transmitted diseases.

Because of the high frequency of infections with both pathogens, a reliably specific and sensitive but at the same time simple diagnostic technique is needed that is suitable for screening, too.

In this work we compared the DNA probe (GP), with the direct immunofluorescence assay (DFA) for the detection of urogenital infections.

Materials and methods

Patients. A total of 162 male and 151 female patients with chlamydia, and 133 male and 121 female patients with gonorrhoeal infection were examined between September 1, 1991 and October 15, 1991 at the STD outpatients' department of the National Institute for Dermato-Venereology, Budapest. Some patients were untreated at the time of specimen collection, others came for a posttreatment check-up.

Urethral and cervical specimens were collected with the swabs supplied with the GP specimen collection system. The same swabs were used for sample taking for DFA. Samples for culturing

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N. gonorrhoeae were taken with platinum loops and put directly on plates. Before taking specimens excess mucus was removed from the cervix with a cleaning swab. The collecting swab was inserted 1–1.5 cm into the endocervical canal, 2–3 cm into the urethra and rotated for 10–30 s then inserted into the GP transport tube or a smear was prepared. Specimens were stored for 0.5 h to 5 days (average 1 day) at 4 °C or at room temperature until the test was performed.

Tests were made in groups of 10–16 according to the direction for use separately for *C. trachomatis* and *N. gonorrhoeae*. After sample preparation, hybridization and separation we detected the chemiluminescent response of acridinium ester-labelled DNA–RNA hybrids in a LEADER 1 luminometer. The cut off value was calculated automatically by adding 300 RLU (relative light unit) in case of *N. gonorrhoeae*, 350 RLU in case of *C. trachomatis* to the actual average RLU of 3 negative controls; results under this value were considered positive and negative, respectively.

C. trachomatis DFA was performed with Chlamyset Antigen FA (Orion Diagnostika, Espoo, Finland) according to the direction for use. Because of technical obstacles out of 313 cases DFA was made in only 252. Before culturing for *N. gonorrhoeae* a Gram-stained smear was prepared in each case. *N. gonorrhoeae* was cultured on a modified Thayer-Martin selective medium for 48 h in 5% CO₂, at 35 °C. Colonies of oxidase-positive Gram-negative diplococci were identified with carbohydrate tests and antibiograms were also prepared.

Results and discussion

Out of 313 patients in males 25.9% (42 of 162) in females 19.9% (30 of 151) were positive for *C. trachomatis*. Comparative data for men show that out of 41 patients examined with GP only 26 were positive also with DFA (63.4%). In women out of 28 only 14 were positive with both tests (Table I).

Out of 254 patients 12% (16 of 133) males, 7.4% (9 of 121) females were positive for *N. gonorrhoeae*. In men the sensitivity was 100%, the specificity 98%; in women these values were 100% and 97.4%, respectively. We found no cases when DFA or culture was positive but GP negative (Table II). Comparison of GP and Gram-stained smear is presented in Table III.

According to literary data the GP technique in comparison with culturing showed 92.5% sensitivity and 99.6% specificity in case of *C. trachomatis* [1–3], while 95.4% sensitivity and 99.8% specificity in case of *N. gonorrhoeae*. In our results significant differences were found only in comparison with DFA – the latter gave a lower positivity. Data in literature show 80–90% positivity rate in GP-positive cases with DFA. We found only 58% correlation.

Table I

Comparison of the results of GP and DFA for detecting *C. trachomatis*

		DFA	
		+	–
Men	GP +	26	15
	GP –	–	86
Women	GP +	14	14
	GP –	–	97

Table II*Comparison of GP and culture in detecting N. gonorrhoeae*

		Culture	
		+	-
Men	GP +	14	2
	GP -	-	117
Women	GP +	6	3
	GP -	-	112

Table III*Comparison of GP and Gram-stained smear in detecting N. gonorrhoeae*

		Smear	
		+	-
Men	GP +	13	3
	GP -	-	117
Women	GP +	2	7
	GP -	-	112

A further advantage of GP is that it gives an opportunity to test two pathogens in one specimen. The sample is transportable for 7 days between 2-25 °C. The entire testing procedure takes two and a half hours.

According to our preliminary data GP Pace 2 assay appears to be suitable also for serial examinations and is an appropriately sensitive and specific method.

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**ANNUAL MEETING
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BACTERIOLOGY

K. CSISZÁR

Demonstration of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* with "gene probe"

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The Gene Probe PACE 2 system (San Diego, Calif.) is a time-saving (2 hours) non-isotopic method of DNA hybridization method. It enables direct demonstration of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in the same sample. Of 314 urogenital and 11 other samples examined for *C. trachomatis* in the period from May, 1992 to April, 1993 33 samples (10.5%) proved positive. Of 179 tests made in the same period for *N. gonorrhoeae* 21 were positive (11.7%). Both pathogens were present in eight of STD materials. The method is easy to perform and allows 7-day transport.

J. DEÁK, A. VIROLAINEN, M. LEINONEN and P. SAIKKU

Detection of *Chlamydia pneumoniae* with polymerase chain reaction

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Chlamydia pneumoniae is the newly recognized third species of the genus *Chlamydia*. It has been shown to be responsible for 10% of pneumonias, and causative

agent of bronchitis, pharyngitis, and flu-like diseases. This organism has recently also been associated with endocarditis, myocarditis and arteriosclerosis. Excellent tests have been introduced for the serodiagnosis of the bacterium, as MIF and LPS ELISA. To surmount the difficulties of cultivation, we developed a PCR method. Primers, which amplify segments of the MOMP gene, were synthesized in oligonucleotide synthesizer. The external genus specific sense primer contains 20, the antisense primer 21 bases. The inner species specific sense primer contains 26, the antisense primer 24 bases. The concentration of primers, dNTP and Mg^{++} as well as the thermal cycling parameters were optimized, Helsinki 12 and Kajani 6 were used as model strains. Chlamydiae were purified and concentrated on Renographin density gradient after cultivation in HL cells. Different quantity of purified and concentrated bacteria were diluted in human sera, lyzed and the DNA extracted with phenol-chloroform-isoamylalcohol. The genus specific PCR detected 100–500 fg and the nested PCR less than 10 fg chlamydial DNA.

E. NAGY and I. SZŐKE

**Comparison of the results obtained with the "TOX-A"
ELISA method and the cytotoxic assay for detection
of *Clostridium difficile* toxin**

Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary

Toxin-producing *Clostridium difficile* is considered to be the leading causative agent of pseudomembranous colitis and antibiotic-associated diarrhoea. It has recently become evident that, as a nosocomial pathogen, *C. difficile* can be responsible for outbreaks of diarrhoeal diseases in different wards. As toxin "A" is responsible for the clinical signs of *C. difficile*-associated infections, it is mandatory to detect the toxin in culture supernatants or faecal samples. The aim of the present study was to evaluate the results obtained with the "Tox-A" (TechLab) ELISA method and quantitative cytotoxic assay. Eighty-five faecal samples and 90 *C. difficile* culture supernatants were tested – 88% of all tests agreed as concerns positive or negative results. In 12% of the tests, the OD values of the ELISA method were just below the cut-off level, showing indefinite results. In these samples, the cytotoxic assay exhibited negative or low titre positive reactions. For the positive samples, the quantitative cytotoxic assay results and the OD values of the ELISA method correlated well.

E. NAGY, C. KRANZ and H. WERNER

**Influence of *Bacteroides vulgatus* on growth
and toxin production of *Clostridium difficile***

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary,
and Department of Clinical Microbiology, Hygiene Institute, University of Tübingen, Germany*

Clostridium difficile can be found in the intestines of about 50% of all healthy sucklings. The composition of the intestinal bacterial flora varies in the course of life; in adults *Bacteroides vulgatus* is the predominant species. During the present study, the influence of *B. vulgatus* strains was investigated on the growth and toxin production of *C. difficile*. Six *B. vulgatus* and 5 toxin-producing *C. difficile* strains were incubated alone or in mixed cultures in brain-heart infusion broth, and colony counts were determined after 48 h. Selective media were used for those cultures where *B. vulgatus* and *C. difficile* were incubated in the same broth. The toxin was determined quantitatively in parallel on the filtered culture supernatants. The Hep-2 cell line was used and the cytotoxic effects of the culture supernatants were determined after incubation for 24 h. As compared to pure cultures of *C. difficile*, the numbers of colony-forming units of all strains tested were at least two log 10 less when they were cultured in the presence of 4 of the 6 *B. vulgatus* strains used. The growth patterns of the *B. vulgatus* strains were not changed in the presence of *C. difficile*. The quantity of toxin produced by the *C. difficile* strains was likewise significantly reduced. The results show that some *B. vulgatus* strains inhibit the growth and toxin production of *C. difficile*. The change in the intestinal bacterial flora of newborns to that observed in adults might therefore explain the inhibition of the primarily established *C. difficile*.

Á. MÉSZÁROS-HARDI, K. EGYHÁZI, Cs. HARGITAI, M. KÁDÁR, Cs. BOGNÁR,
Zs. SENONER and J. TINN

Examination of karstic water in Tapolca, 1991-92

Department of Animal Physiology and Health, University of Agricultural Sciences, Gödöllő, Hungary

We took samples on 19 occasions from 50 sites of underground karstic cave water (Lake cave, Hospital cave) and from a surface karstic well, the Mill pond. The analysis of samples was carried out by bacteriological and chemical methods. In 1991, we mapped the location of the polluted water that arrives in both caves at two sampling points. As we continued the investigation in 1992, we confirmed that it was likely that urban sewage water was the most important of the potential source of pollution. Furthermore, we found that there were sharp seasonal changes in the level of water contamination during the examination period. Despite this fluctuation, there was no occasion when all the karstic cave water samples were of drinking water quality. In addition to the cave water, the Mill pond water was also regularly contaminated.

B. DÉNES, L. TEKES and I. HAJTÓS

**Eradication of *Brucella ovis* infection in breeding ram
flocks of Carpathian Ruthenia (Ukraine)**

*Carpathian Veterinary Service, Veterinary Laboratory of Técső, Ukraine, Central Veterinary Institute,
Budapest, Hungary, and Veterinary Institute of Miskolc, Hungary*

In order to eradicate *Brucella ovis* infections, rams were clinically examined and serologically tested by agar-gel precipitation (AGP), by the cool variant of complement fixation (CF) tests and by ELISA every 30 to 40 days. At the beginning of the investigations, the ratio of seroconverted breeding rams detected with the three tests were 25.7%, 17.9%, 28.6%, 20% and 13.5%, respectively, in flocks I to V. When ELISA was also used, three to four consecutive tests were needed to obtain the seronegative (infection free) state in flocks I, III and V, and 6 tests in flock II. The eradication was made only by AGP and CF tests in flock IV that needed 7 consecutive tests to be done. Proven to be infected in all of the three serological tests, rams were detected by ELISA in 95.1%, while the AGP and CF tests detected only 79.5% and 41.0%, respectively.

Raising of replacement ram lambs isolated from the ewes and rams, as well as isolation of virgin and older breeding rams during the mating season have been considered essential to prevent the spreading of *B. ovis* infection within the flock.

It has been pointed out that *B. ovis* infection can be eradicated within a relatively short period by systematical examination of flocks in question by culling the diseased and seroconverted animals, and correct management (isolated raising of virgin and breeding rams) without the simultaneous screening of ewes.

E. NAGY, B. MANNCKE and H. WERNER

**Investigation of fibronectin and vitronectin binding
of *Bacteroides* strains by latex agglutination test**

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary,
and Department of Medical Microbiology, Hygiene Institute, University of Tübingen, Germany*

Different species of the genus *Bacteroides* are predominant in the human intestinal flora. The species most frequently isolated from faecal samples are *B. vulgatus* and *B. thetaiotaomicron*, whereas *B. fragilis* is the leading anaerobic pathogen in human infections developing after abdominal or gynaecological surgery. Both in pathological processes and in the establishment of the members of the normal flora on mucosal surfaces, the binding of the bacteria to physiological glycoproteins is well documented. The aim of the present study was to investigate the fibronectin and vitronectin bindings of 152 strains belonging in 9 *Bacteroides* species of different origins (clinical materials or faeces) by means of latex agglutination. Twenty-three per cent of the strains isolated from faeces exhibited fibronectin binding, as did 46% of the

strains obtained from severe infections. Most of the isolates displaying fibronectin binding belonged in the species *B. fragilis* or *B. vulgatus*. The binding could be inhibited by preincubation of the cells with an excess amount of fibronectin. Vitronectin binding was less common, but was always observed in parallel with fibronectin binding.

I. GADÓ, I. TÓTH, J. PÁSZTI, I. BARCS, V. G. LÁSZLÓ, É. CZIRÓK,
M. HERPAY, B. NAGY and H. MILCH

**Preparation and use of DNA probes in *Escherichia coli*,
Shigella and *Salmonella* strains**

*B. Johan National Institute of Hygiene, Budapest, and Veterinary Medical Research Institute of the
Hungarian Academy of Sciences, Budapest, Hungary*

Twelve DNA probes suitable to demonstrate certain pathogenicity genes in *Escherichia coli*, *Shigella* and *Salmonella* strains were prepared; 7 probes were adequate to demonstrate various enterotoxin and cytotoxin genes, 3 for adhesion factors and 2 to detect the presence of plasmids characteristic of pathogenicity groups EHEC and EIEC. To prepare these probes, fragments, containing the specific sequences were isolated from clones received from abroad and ³²P-labelling was performed with random priming method. The utility of the probes was verified with colony hybridization. The correlation between this method, the biological toxin test and the in vitro adhesion assay was examined. Furthermore, the occurrence of toxin and invasion genes homologous with our probes was surveyed among *E. coli*, *Shigella* and different *Salmonella* sero- and phagetypes.

A. LIPCSEY and A. SZENTMIHÁLYI

**Epidemiological typing of *Pseudomonas aeruginosa*
by PcmTox probe and pulsed field gel electrophoresis**

B. Johan National Institute of Hygiene, Budapest, Hungary

The hypervariable region of the exotoxin A structural gene may be utilized in a genprobe (U-probe) for the typing of *Pseudomonas aeruginosa* after genomic digestion with Sal I, Bgl II, Xho I and Southern blot. This method combined with the pulsed field gel electrophoresis – that makes possible to separate the largest DNA molecules – yields a genetic fingerprint which is more reliable and reproducible than most of the phenotype methods. Twenty-three *P. aeruginosa* 011 strains isolated from a Perinatal Intensive Care Unit – which were very similar in their pyocin types and phage patterns – were distinguishable using this method.

L. EMÖDY, H. FENYVESI and T. TOMCSÁNYI

The role of alpha-haemolysin in *Proteus penneri* virulence

Department of Microbiology, University Medical School, Pécs, and Department of Zoology, Janus Pannonius University, Pécs, Hungary

The virulence of a *Proteus penneri* strain causing unusually large zone of haemolysis on blood agar plate was studied. The haemolysin, similarly to *Escherichia coli* and *Morganella morganii* alpha-haemolysins, proved to be filterable in liquid cultures. To study the role in virulence of the filterable haemolysin of *P. penneri* in vitro and in vivo assays have been conducted with the wild type strain and its non-haemolytic transposon mutants. On HeLa, Hep-2 and Vero cell cultures the cytotoxic ED₅₀ values for the wild type strain were hundred times lower than those for the non-haemolytic mutants. The filterable haemolysin proved to be a decisive factor of virulence in mouse models (intraperitoneal, intravenous and intranasal infection), and in allantoically infected chick embryos. Pathological findings indicated that acute toxic effects of the haemolysin are substantially involved in the pathogenesis.

T. PÁL, AN LI, PHUNG DAC CAM and A. A. LINDBERG

Immune responses of dysenteric patients and vaccinated volunteers against various *Shigella* antigens

Department of Clinical Bacteriology, Huddinge, Sweden, and Department of Microbiology, University Medical School, Pécs, Hungary

Bacillary dysentery patients from Vietnam (*Shigella flexneri* 1b n=9; *S. flexneri* 2a n=12 and *S. dysenteriae* 1 n=10) and from Sweden (*S. flexneri* n=9), as well as volunteers vaccinated orally with a Δ aroD *S. flexneri* Y vaccine strain (SFL124) were tested for their peripheral humoral immune responses against lipopolysaccharide (LPS) and invasion plasmid coded protein antigens (Ipa). The goal of the study was to determine which parameter of the immune response is suitable to support the clinical diagnosis of bacillary dysentery or monitor the effect and antigen delivery during vaccination. Both Vietnamese and Swedish patients exhibited significant titre increases ($p < 0.05$) against the LPS antigen in the IgA (postinfection days 7, and 30) and in the IgG classes (postinfection days 7, 30, 90, and 180) as compared to the corresponding values of the local healthy population. Vietnamese patients showed a rather uniform, but moderate response against Ipa in the IgG class. In contrast, the corresponding response in Swedish patients showed high individual variations but still were significant ($p < 0.05$) in the IgA (day 7), as well as in the IgG class (day 30). Vaccinated volunteers exhibited a significant response against LPS after a single vaccine dose. However, even after three repeated doses, only those volunteers responded with Ipa-specific titre increases who had been primed for these antigens by previous *Shigella* or enteroinvasive *Escherichia coli* infection. On the basis of these

findings we conclude that for supporting the clinical diagnosis, as well as for following the immune stimulation in vaccine trials the examination of LPS specific responses is more suitable than that of the Ipa-specific one.

I. BARCS and J. PÁSZTI

**Characterization of enzymes inactivating penicillins,
aminoglycosides and lincosamides determined by
Staphylococcus epidermidis plasmid pBI109PGL**

B. Johan National Institute of Hygiene, Budapest, Hungary

The 44 kb *Staphylococcus epidermidis* plasmid pBI109PGL determines resistance to penicillins, aminoglycosides, and lincosamides. The penicillin resistance is due to production of an inducible type V beta-lactamase, the aminoglycoside resistance to an aminoglycoside 6' acetyltransferase V, and resistance to the lincosamides to a lincosamide inactivation nucleotydilation enzyme A'. By Southern blotting, a 13 kb XbaI fragment harboured the linA' gene. The same fragment was found to determine aminoglycoside resistance. Examination of deletion mutants suggested aminoglycoside resistance gene to be joint the region responsible for regulation of beta-lactamase production.

H. KOVÁCS-DOMJÁN and A. FÁBIÁN

**Rapid methods for identification of *Listeria*,
with special regard to gene-probe**

National Food Investigation Institute, Budapest, Hungary

The conventional cultivation method for detection and confirmation of *Listeria* from foods is a time-consuming and laborious procedure. It takes 7-12 days that makes difficulties e.g. in case of short deadlines in export activity. To reduce the time required, rapid methods have been developed and are available commercially. Three of them have been tested in our laboratory: (1) Micro-ID *Listeria* System (Organon Teknika); (2) API *Listeria* System (bio Mérieux). Both techniques are based on biochemical tests by detection of enzymes and metabolic end-products. (3) Accu Probe *Listeria monocytogenes* culture identification test (Gen-Probe) based on the principle of gene hybridization. Twenty strains were tested with each of methods (1) and (2). Micro-ID gave correct results in 100% of the cases and in 4 h indicated the presence of *L. monocytogenes*. In contrast to API test, the results – observable after 24 h – were false in 20%. About 100 samples were tested with Accu Probe. This method has proved to be very specific (100%). The procedure for the analysis of 20 samples took 1 h only.

L. MOLNÁR

**Sensitivity of *Serpulina hyodysenteriae*
to chemotherapeutics**

Department of Microbiology and Infectious Diseases, Veterinary University, Budapest, Hungary

Swine dysentery (SD) can persist for decades in large-scale pig herds. In these herds it is necessary to treat the pigs systematically. The result of the permanent treatment is that resistance develops sooner or later against different chemotherapeutics. The following chemotherapeutics have been widely used during the last one and half decade in Hungary for the prevention and therapy of SD: tylosin, carbadox, dimetridazole, lincomycin, monenzin, sedecamycin. These drugs, with exception of the two last ones, are distributed world-wide for the control of the disease. The degree of sensitivity of *Serpulina hyodysenteriae* strains to the chemotherapeutics mentioned above is different and resistance-relations also have developed differently. Carbadox and sedecamycin resistant strains have not been isolated till now. The susceptibility of the strains to dimetridazole has notable decreased nearly half of the strains are only "moderately sensitive". In the majority of the strains resistance has developed against tylosin which earlier had an excellent effect. About half of the strains is sensitive to lincomycin in our days. Among the antibiotics tiamulin is the most effective recently but some resistant strains have already appeared.

É. MOLNÁR and V. FUSSING

**DNA patterns of *Actinobacillus pleuropneumoniae*
biotype-1 serotype 2 strains in Hungary**

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and National Veterinary Laboratory, Ministry of Agriculture, Copenhagen, Denmark*

Twelve serotypes of *Actinobacillus pleuropneumoniae* biotype-1 (V-factor dependent) including subtypes A and B of serotype 5 have been identified in Hungary with the predominance of serotype 2. The aim of the present work was to compare the restriction patterns of different serotype 2 strains to find an explanation for the difference of the virulence observed earlier and the haemolysin and cytotoxin described by other workers. DNA-DNA hybridization was carried out with 30 *A. pleuropneumoniae* serotype 2 strains isolated from 22 farms in Hungary. Whole-cell DNA was prepared from suspensions after treatment with proteinase K and SDS, it was purified with phenol and phenol-chloroform extraction. The total bacterial DNA was cleaved by one of eight restriction enzymes and electrophoresed in agarose. The fragments were transferred to a nylon membrane and hybridized to digoxigenin-labelled nucleic acid probes. The Hind III, restriction patterns of the 30 strains showed 8 different groups. The homology of the strains proved to be high (72–94%).

Cs. MOLNÁR, Zs. HEVESSY, A. FENYVESI, I. FRANCIA, T. MOLNÁR
and F. ROZGONYI

**Effect of neutron irradiation on experimental
Staphylococcus epidermidis infection in BALB/c mice**

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Debrecen, Hungary*

Three doses (1.57; 2.45; 3.83 Gray) of neutron irradiation were given to BALB/c mice three days prior to intraperitoneal (i.p.) infection with a *Staphylococcus epidermidis* strain at $1 \times$, $4 \times$, $16 \times$ and 64×10^6 c.f.u./g body wt suspended in dextran microcarrier solution. Lethality rates and organ persistence of cocci were in direct correlation with the amounts of bacteria injected. Less organ persistence occurred in mice received low dose of irradiation than in non-irradiated mice at the same bacterial challenge. In contrast, there was a 2.5-fold persistence in mice irradiated with LD₅₀ neutron dose than in the non-irradiated ones. There was no difference between females and males in the lethality rates. Half of the survivors was overanaesthetized and dissected 10 days after the challenge. The number of splenomegaly cases and the size of spleens also enhanced in the irradiated mice. The organ persistence of the cocci was not more frequent in mice received low neutron dose, although the lethality rate was significantly higher at a challenge of 16×10^6 c.f.u./g body wt. The extremely large spleens and the atrophic spleens found in mice irradiated with high doses of neutron radiation were probably attributable to radiation injury, since the organ persistence was also the highest in these mice. It is concluded that a high dose of neutron irradiation renders the mice fatally susceptible to a low amount of *S. epidermidis* by i.p. challenge. This fact calls attention to the risk of coagulase-negative staphylococcal infections in human irradiation therapy.

É. B. JÁKICS, J. SZABÓ, K. DARVAS, I. PULAY, A. SZENTMIHÁLYI
and M. KONKOLY THEGE

**Epidemiological characterization of *Pseudomonas*
aeruginosa isolates from an Intensive Care Unit of surgery**

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Semmelweis University Medical School, Budapest, Hungary*

In the Intensive Care Unit of the First Department of Surgery, multiply resistant *Pseudomonas aeruginosa* occurred frequently. In 1992 1031 samples from 175 patients were cultured. *P. aeruginosa* was isolated from 86 patients, 152 out of 388 strains in pure cultures. From the beginning of 1993 distribution of serotype, pyocin type, phage pattern and antibiotic sensitivity pattern was examined. During this period, 52 *P. aeruginosa* strain were isolated from samples of 16 patients, 12 strains in pure cultures.

By the examined characters all but two patients, strains differed from those that colonized other individuals. Twelve isolates of 6 patients were resistant to new fluoroquinolones.

Zs. CSUKÁS, M. PÁLFALVI and R. KISS

Eye injury with bacterial complication

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A serious, cornea-penetrating injury was caused by a metal foreign body to a 16 years old boy during his practice in the workroom. The place of the foreign body was localized by X-ray and ultrasonic examination in the posterior part of the vitreous body, near to the retina. At admission, the basic eye wound toilet and the closure of the corneal wound was performed. General and subconjunctival broad-spectrum antibiotic therapy was started. After 15 h, an endophthalmitis developed, which was demonstrated by ultrasonic examination as gas production and exudate in the vitreous body. In this time, the foreign body was removed by a vitrectomy and an anaerobic, non-spore forming microorganism, *Bifidobacterium adolescentis* was isolated from the washing material of the vitreous body. The vitrectomy and appropriate modification of antibiotic therapy prevented the spread of the inflammation but the visus of the injured eye was lost.

É. ÜREGOVÁ, K. CSISZÁR and J. MOLNÁR

The elimination of different antibiotic resistance plasmids from *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* by promethazine

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Promethazine induced antibacterial effect on 12 strains of *Yersinia enterocolitica* and two strains of *Yersinia pseudotuberculosis*. There was no difference in the minimal inhibitory concentrations of the isolates of various origin. Antibiotic resistance of some the *Y. enterocolitica* strains (Ap, Str, Gm) was eliminated at various frequency from different strains. The *Y. pseudotuberculosis* strains were less sensitive to the plasmid curing effect of promethazine than *Y. enterocolitica*. The curing effect of promethazine was not dependent of on the serotype of yersinia isolate.

N. PAPP-JUHÁSZ, J. MOLNÁR and L. EMŐDY

Elimination of haemolysin plasmids from *Escherichia coli* strains

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A. Szent-Györgyi University Medical School, Szeged, and Institute of Microbiology,
University Medical School, Pécs, Hungary*

The promethazine and structurally related imipramine were able to eliminate the haemolysin plasmids of *Escherichia coli* CW 22 and from *E. coli* CW 35 strains. The HLy, Tc plasmid (110 Md) was more stable in J53 and HB101 strains than in the CW strains. Promethazine had lower MIC values for cured strains than for parent strains. Promethazine can contraselect plasmidless cells in a population above a certain concentration. The action of bacterial haemolysin was not inactivated in the presence of promethazine and imipramine.

J. MOLNÁR, N. BATHÓ, V. CSIK, J. CHEVALIER and A. CREMIEUX

Interaction between tricyclic psychopharmacocons and some antibiotics

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The tricyclic psychopharmacocons cure plasmids and inhibit plasmid transfer among bacteria due to the inhibition of supercoiling activity on DNA gyrase. An interaction was found between clozapine, imipramine, promethazine and penicillins including ampicillin in vitro. The nature of interaction is based on a charge transfer complex, which is formed between clozapine, imipramine, promethazine and penicillins. Ampicillin reduced the highest energy peaks of clozapine, promethazine and imipramine in differential spectrophotometric study. Streptomycin was not able to alter the spectrum of clozapine, however, tetracycline reduced somewhat all the peaks of clozapine. Clozapine and promethazine exerted synergistic effect with ampicillin and gentamicin on *Escherichia coli* cells. This kind of interaction was weak with tetracycline and was missing in the case of imipramine.

J. PÁSZTI, I. GADÓ, I. BARCS, M. HERPAY, B. NAGY and H. MILCH

Comparison of EIEC-, VT1- and AIDA specific DNA probes labelled with ^{32}P isotope and digoxigenin

B. Johan National Institute of Hygiene, Budapest, and Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

A total of 50 different enteropathogenic *Enterobacteriaceae* isolates were examined for the presence of EIEC, VT1 and AIDA-I genes parallel with specific gene probes labelled with ^{32}P isotope and digoxigenin. EIEC specific probe was used to examine *Escherichia coli* strains of human and animal origin, belonging to 9 serogroups, isolated from Hungary and Tengiz. Out of the examined 18 strains 15 were positive of serogroups 0112, 0124, 0136, 0143, 0147, 0152, 0164, 2 of serogroup 0136 and 1 of 028 were negative with both methods. Identical results were obtained by parallel testing with VT1-specific probes of 30 *E. coli* strains belonging to different serogroups, 1 *Shigella dysenteriae* 1 and 1 *Klebsiella pneumoniae* strain. The data demonstrated the effectivity of the biological examinations. To demonstrate diffuse adhesion (AIDA-I) positive and negative control strains were used; the two methods gave identical results with both labelling methods. On the basis of our examinations the non-isotopic hybridization and radioactive methods were of the same value, so non-isotopic method was suitable to demonstrate virulence factors of enteropathogenic strains and a quicker and simpler tool to characterize these bacteria.

M. HERPAY, É. CZIRÓK, E. GYÖRGY, G. HÉRMAN and J. PÁSZTI

Occurrence of enteropathogenic toxin producing *Escherichia coli* bacteria in Hungary

B. Johan National Institute of Hygiene, Budapest, and Budapest Institute, National Public Health Service, Budapest, Hungary

Toxin production of 1403 *Escherichia coli* strains and other *Enterobacteriaceae* (*E. coli* 14, *C. freundii* 1) collected between 1989–1993 was analyzed. Fifteen out of 1047 (*E. coli* 14, *C. freundii* 1) produced heat labile enterotoxin (LT), 7 of them were isolated from travellers returning from Mongolia, Bangladesh and Tengiz. Of 161 strains examined for heat stable enterotoxin (ST) 28 were ST⁺ (*E. coli* 27, *C. freundii* 1). In 21 cases ST⁺ strains were isolated from patients returning from Tengiz or Sweden, the remainders were isolated from cases infected in Hungary. Hungarian enterotoxin producing strains originated from sporadic (*E. coli* 06, 07, 015, 020, 0159, *C. freundii* LT⁺ and *E. coli* 020 ST⁺) and epidemic (*E. coli* ONT,Sp., *C. freundii* ST⁺) cases, respectively. Verotoxin (VT) production was examined in 195 strains (189 of them *E. coli*). Three VT⁺ *E. coli* (026:H11) were connected with an epidemic or family outbreak (0157:HNT).

E. NAGY, Zs. KÓCZIÁN, E. DÓSA, Á. VÁRKONYI and E. HAJDÚ

**Evaluation of bacteriological results obtained with
nasopharyngeal swab sampling in upper respiratory tract
infections**

Department of Clinical Microbiology, Department of Pediatrics, A. Szent-Györgyi University Medical School, and Department of Infectious Diseases, County Hospital, Szeged, Hungary

Upper respiratory tract infections caused by different bacteria are very common in children. For bacteriological examinations, throat or nasal swabs are usually employed in Hungary, whereas nasopharyngeal swabs are often used for this purpose in other countries of Europe. During a 5-month study, nasopharyngeal swabs with transport media and conventional throat swabs without transport media were used in parallel for sampling in children with upper respiratory tract infections. Out of 114 cases, 48 children were negative with both methods, 32 children had a positive culture with the nasopharyngeal swab, whereas the material obtained with the throat swab proved negative. In 12 children, pathogenic bacteria could be isolated only from the throat swab. Both sampling methods resulted in positive cultures in 22 children. *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella (Branhamella) catarrhalis* were isolated more frequently from the specimens taken with nasopharyngeal swabs and forwarded into the laboratory in transport media.

E. DÓSA and E. NAGY

**Evaluation of rapid latex agglutination test for detection
of group B streptococci in the vagina**

Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary

Group B streptococci (*Streptococcus agalactiae*) have been found to be the most frequent cause of neonatal infections during the first months of extrauterine life. Two different syndromes, involving the early and the late onset of the infections, are known, manifested as meningitis and bacteraemia or as respiratory distress. The vaginal colonization of *S. agalactiae* is relatively common among women during pregnancy. Although newborn children acquire *S. agalactiae* from the birth canal, it is presumed that the main reservoir of the bacteria is the gastrointestinal tract. Most women carry *S. agalactiae* in the vagina intermittently during life, so it may therefore be important to determine the carriage rate during pregnancy by a direct rapid method just before the delivery. In the present study the vaginal carriage rate of *S. agalactiae* in women attending an outpatient clinic was investigated. Specimens were obtained from 110 women by means of paired vaginal swabs and by vaginal washing using 1 ml of sterile physiological NaCl solution. One swab and the vaginal washing were cultured semi-quantitatively or quantitatively. Direct antigen detection of *S. agalactiae* was carried out by the Streptex (Wellcome Diagnostic) using the second swab and the vaginal

washing parallel. Out of the 110 vaginal samples taken with swabs 20 proved to be *S. agalactiae*-positive by the culture method and by the direct antigen detection method. Two false negative results were obtained by the direct antigen detection method when the vaginal washings were used. The quantitative determination showed that all specimens where the colony counts of *S. agalactiae* were higher than 10^2 /ml exhibited a positive reaction in the antigen test, while those in which less than 10^2 colonies/ml were present, proved to be negative. The direct latex agglutination test seems to be an adequate method to screen *S. agalactiae* colonization of women before delivery.

K. CSISZÁR, A. SZENTMIHÁLYI, A. LIPCSEY, J. FÖLDES, M. E. KAUFMANN
and T. L. PITT

Analysis of Hungarian multiresistant *Pseudomonas aeruginosa* 012 strains

Nógrád County Institute, National Public Health Service, Salgótarján, B. Johan National Institute of Hygiene, Budapest, Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary, and Division of Hospital Infection, Central Public Health Laboratory, London, UK

Between 1990 and 1992 we isolated 46 strains of *Pseudomonas aeruginosa* serogroup 012 from clinical specimens of patients. No clustering of cases was observed. All isolates were resistant to antipseudomonal antibiotics, with the exception of imipenem and produced the same chromosomal broad-spectrum beta-lactamase. All isolates were pyocin type 1/h and SDS-PAGE profiles of whole cell proteins were similar. Ten strains were selected and examined further by gene probing with the pCMtox plasmid containing the *Exo A* structural gene of *P. aeruginosa* and the variable upstream region. Southern blots of digests of DNA showed that all Hungarian 012 isolates were genotypically identical and were also indistinguishable from the strain reported to be prevalent in various parts of Europe. They were, however, distinguishable from some English isolates. In conclusion, Hungarian multiresistant *P. aeruginosa* of serotype 012 share clonal properties with other European and Near Eastern strains suggesting a common ancestral lineage.

M. KÁLMÁN

The antibacterial activity of a new carbapenem antibiotic

Csongrád County Institute, National Public Health Service, Szeged, Hungary

In an international multicentre Meropenem study our laboratory isolated 14 aerobic bacteria from 26 samples (blood, trachea, urine) received from the Children's Hospital. The antibacterial activity of Meropenem, Imipenem and Cefotaxim was examined by zone diameters on Mueller-Hinton agar (64 strains) and by MIC breakpoints with microbroth dilution on Sensititre-plates from ICI (6 polyresistant

strains). The provisional QC limits were determined by ATCC reference strains of ICI. Only 1 *Enterococcus faecalis* and 1 *Enterococcus faecium* were resistant to Meropenem, Imipenem and Cefotaxim – showing zero zone diameter and a 16 µg/ml MIC break-point. A *Serratia marcescens* strain from urine was sensitive only to Meropenem, Imipenem and Ciprofloxacin. This parenteral carbapenem antibiotic seems to have an exceptionally broad spectrum of antibacterial activity. It is less toxic than Imipenem and is useful chiefly in children's hospitals.

CS. BOGNÁR, Á. HERENDI, GY. CORRADI, R. DÉNES, T. MAJOR SR.
and R. SZLÁVIK

Antibiotic resistance in different hospital wards

*B. Johan National Institute of Hygiene, Department of Urology, and First Department of Surgery,
Semmelweis University Medical School, Institute of Haematology and Blood Transplantation, and State
Railways Hospital, Budapest, Hungary*

Bacterial species most frequently isolated between January 1, 1990 and December 31, 1992 were studied for antibiotic sensitivity. Figures in parentheses represent sensitivity in per cent of isolates at various wards. *Pseudomonas aeruginosa*: carbenicillin (25/50/52/8), azlocillin (60/71/77/17), mezlocillin (16/14/40/7), ceftriaxone (2/14/50/9), tetracycline (18/50/30/20), gentamicin (28/71/70/54), tobramycin (30/100/70/50), netilmicin (98/71/95/71), amikacin (88/86/96/100). *Klebsiella oxytoca*: mezlocillin (36/27/10/27), cefalexin (90/100/92/50), cefamandole (90/90/92/50), cefuroxim (90/100/92/51), ceftriaxone (100/90/92/73), tobramycin (90/90/98/51).

Klebsiella pneumoniae: cefalexin (66/90/64/32), cefamandole (65/98/64/35), cefuroxim 71/90/67/41), tetracycline (64/100/64/49), netilmicin (87/100/81/64).

Staphylococcus aureus: oxacillin (67/87/77/58), cefalexin (67/88/75/58), cefamandole (67/90/75/53), cefuroxim (67/90/75/51), ceftriaxone (66/90/70/50), netilmicin (66/100/100/77), erythromycin (75/88/88/56). The data for antibiotics showing no significant differences are not presented. That the susceptibility of bacteria isolated from various wards differ significantly, it emphasises the necessity of accurate and adequate susceptibility examinations and antibiotic therapy based on them.

T. MAJOR SR., CS. BOGNÁR, Á. HERENDI, B. LÁNYI and K. EGYED

Resistance to antibiotics at a pulmonology department

State Railways Hospital, and B. Johan National Institute of Hygiene, Budapest, Hungary

The bacteriological examination of respiratory specimens and the evaluation of the results presents many problems, and, accordingly empirical therapy is usual. We present the antibiotic sensitivity of 4777 bacterial strains isolated most frequently from

sputum samples in the last three years. The data are compared with the country-wide data for 1991.

The percentage distribution of sensitive isolates was as follows (country-wide data for 1991 in parentheses). *Streptococcus pneumoniae*: penicillin 83 (63), tetracycline 50 (44), co-trimoxazole 50 (33), pefloxacin 67 (11.4), ciprofloxacin 100 (73). *Staphylococcus aureus*: penicillin 100 (97), oxacillin 78 (94), clavulanic acid – amoxicillin 100 (96), pefloxacin 86 (90), ciprofloxacin 100 (95). *Haemophilus* spp.: clavulanic acid – amoxicillin 100 (96), pefloxacin 89 (98), ciprofloxacin 100 (94). Sensitivity of all other bacteria agreed closely with the country-wide data.

Á. HERENDI, Cs. BOGNÁR, R. SZLÁVIK and T. MAJOR SR.

Sensitivity of anaerobic bacteria isolated in the years 1990–1993

B. Johan National Institute of Hygiene, First Department of Surgery, Semmelweis University Medical School, and State Railways Hospital, Budapest, Hungary

Percentage distribution of antibiotic sensitivity of the most frequently occurring anaerobes was as follows (bracketed figures stand for nation-wide data in 1993 for clindamycin). *Clostridium perfringens*: penicillin 100, cefoxitin 100, tetracyclin 95, clindamycin 100 (92), metronidazol 100. *Peptostreptococcus anaerobius*: penicillin 86, cefoxitin 86, tetracyclin 100, clindamycin 100 (100), metronidazol 71. *Peptostreptococcus asaccharolyticus*: penicillin 92, cefoxitin 92, tetracyclin 67, clindamycin 92 (98), metronidazol 92. *Fusobacterium nucleatum*: penicillin 78, cefoxitin 100, tetracyclin 100, clindamycin 100 (97), metronidazol 100. *Eubacterium lentum*: penicillin 88, cefoxitin 100, tetracyclin 88, clindamycin 100 (100), metronidazol 88. *Bacteroides fragilis*: cefoxitin 96, clindamycin 94 (99), metronidazol 94. *Bacteroides* spp.: penicillin 0, cefoxitin 82, tetracyclin 100, clindamycin 100 (100), metronidazol 100.

T. MAJOR JR., T. MAJOR SR., K. EGYED, Cs. BOGNÁR and Á. HERENDI

Resistance of *Pseudomonas* strains isolated from respiratory specimens

Department of Pulmonology, Semmelweis University Medical School, State Railways Hospital, and B. Johan National Institute of Hygiene, Budapest, Hungary

After the genus *Haemophilus*, *Pseudomonas aeruginosa* is the agent most frequently associated with lower respiratory diseases. In the period from January 1, 1990 to December 31, 1992 *P. aeruginosa* strains were isolated from washed sputum samples of 689 patients. In 1990, 52% of 278 isolates, in 1991 54% of 239 isolates, and in 1992, 75% of 162 isolates proved sensitive to antibiotics used in therapy. It should

be noted that routine tests with a number of new antibiotic agents had not been performed before 1992. No increase in the resistance to aminoglycosides was observed in the period under study. Ceftriaxone sensitivity increased conspicuously during the three-year period (67–86–100%). Incubation of sputum samples at 37 °C overnight and for a day thereafter yielded *P. aeruginosa* strains growing slowly and forming a mucous capsule. These strains, unlike other *P. aeruginosa* isolates, showed in vitro sensitivity to several antibiotic agents.

M. KÁDÁR

Proficiency testing with simulated water samples in Hungary

B. Johan National Institute of Hygiene, Budapest, Hungary

In 1991 the Water Microbiology Laboratory of the National Institute of Hygiene has initiated an inter laboratory proficiency test series on voluntary basis with divided or simulated water samples. Since then three testing have been organized with the aim of gaining information on the uniformity and reliability of routine testing, identifying the possible causes of inappropriate performances, and experience on the way of evaluating the results. In testing the reliability of coliform detection, most of the participants (30–40 per test) presented acceptable results both in determining coliform counts and presence of thermotolerant strains. The most considerable deviations and the highest scattering was found in total colony counts even if a standard culture medium was provided with the samples.

É. ERDŐS

Occurrence of *Aeromonas* bacteria in drinking waters of Csongrád County

Csongrád County Institute, National Public Health Service, Szeged, Hungary

There is a growing body of evidence that the members of the genus *Aeromonas* are potential pathogens, causing a wide variety of human infections. Data indicate that most of these diseases are food- or water-associated, therefore it was decided to examine the occurrence of *Aeromonas* bacteria in drinking water samples in Csongrád county. A total of 651 samples derived from deep wells (213 samples), piped waters (130 samples) and distribution system (298 samples) of public and industrial water works. Enumeration of *Aeromonas* bacteria was made on Ampicillin-Dextrin agar by membrane filtration. Identification was based on colony morphology and different biochemical tests according to the scheme of Popoff. Isolation frequency of *Aeromonas* was higher in the industrial water works than in the public ones, the highest frequencies

being found in supplied water leaving the water works. *Aeromonas* densities exceeded 100 c.f.u./100 ml in more than half of the positive samples originated from the distribution system. Correlation of aeromonas densities with the chemical parameters and the treatment processes of waters was investigated. *A. hydrophila* was the most frequently isolated species but *A. caviae*, *A. sobria* and not identified *Aeromonas* were also found.

VIROLOGY

J. BURGYÁN, T. DALMAY and Á. KOLLÁR

Mapping of cymbidium tombusvirus genes for functions

Agricultural Biotechnology Center, Gödöllő, Hungary

Genomic RNA of cymbidium ringspot tombusvirus (CyRSV) carries five large open reading frames (ORFs). The two 5'proximal frames encode proteins of 33 and 92-kD and the three 3'proximal encode proteins of 41, 22 and 19-kD. In addition, a small reading frame encoding a protein of 4-kD (ORF 6) was hypothesized by computer analysis of the 3'terminal nontranslated region of sequenced tombusviruses (Boyko and Karasev, 1992). ORF 6 potentially encodes a protein of 32-69 amino acids. The function of putative gene products was investigated with the use of several mutants. Mutants in ORF 1 and 2 were not viable indicating that both 33- and 92-kD proteins are required for replication. Deletion of a large portion of the coat protein gene did not prevent replication of viral RNA and cell-to-cell movement, but interfered severely with long-distance translocation. No virus particles or viral RNA could be detected in inoculated or upper leaves of plants inoculated with transcripts obtained from mutants not expressing the 22-kD protein. However, replication and encapsidation occurred normally in inoculated protoplasts indicating that this gene product is transport protein. Conversely, no essential role could be assigned to putative gene products of ORF 5 (19-kD protein) and ORF 6. It was also shown that factors other than gene expression can influence the replication of RNA mutants due probably to instability of RNA molecules.

T. DALMAY, M. RUSSO and J. BURGYÁN

**Repair in vivo of altered 3' terminus of cymbidium
ringspot tomosvirus RNA**

Agricultural Biotechnology Center, Gödöllő, Hungary

Progeny RNA of cymbidium ringspot tomosvirus (CyRSV) in vitro RNA transcripts ending -GGG, instead of -CCC as in wild-type RNA, was polyadenylated and cloned using oligo(dT) as a primer. cDNA clones containing the 3' end were sequenced and shown to revert to wild-type. Mutants were prepared which lacked the three terminal Cs: transcripts were infectious and progeny RNA was in the most part equal to the wild-type. Transcripts ending-GGCCA_n were also infectious and most part of the progeny RNA was equal to the inoculum, indicating that this terminus is compatible with replication without repair, since the unusual G residue at the -3 position was conserved. This result strongly suggests that the minus-strand synthesis initiates at/or downstream of this G residue. Deletion of G at position -4 completely abolished infectivity. These observations suggest that there is a turnover of the viral RNA 3' end which involves removal and resynthesis of the terminus before replication. It is suggested the occurrence of a repair mechanism of the 3' end of CyRSV similar to telomerase activity on chromosomes.

K. SALÁNKI, T. DALMAY and J. BURGYÁN

**Expression of a heterologous viral coat protein gene
by recombinant DI RNA of cymbidium ringspot
tomosvirus**

Institute for Plant Sciences, Agricultural Biotechnology Center, Gödöllő, Hungary

Defective interfering (DI) RNAs of cymbidium ringspot tomosvirus (CyRSV) are mosaic molecules made up of non-contiguous regions of genomic RNA, that are generated de novo during viral replication. We have attempted to prepare a plant viral vector, based on DI RNA molecules. Recombinant DI clones were prepared inserting the coat protein gene of tomato aspermy cucumovirus (TAV) into a 481 bp long DI clone in two different positions. The translation properties of the constructions were examined in rabbit reticulocyte lysate extract. The translational pattern obtained with the transcripts and the authentic TAV RNA 4 were identical. *Nicotiana clevelandii* plants were coinoculated with in vitro transcripts of the hybrid DI RNA clones and the genomic transcript of CyRSV. Northern analysis of progeny viral RNA in the inoculated and systematically infected leaves showed that the constructs are able to replicate in the presence of the genomic RNA, but the stability of the recombinant DI RNAs is dependent on the position of the insertion of the coat protein gene. Western blot analysis showed that the TAV coat protein gene was expressed correctly in the

case of the stable hybrid RNAs. The results of the experiments demonstrate that DI molecules can potentially serve as gene expression vector.

Á. KOLLÁR, T. DALMAY and J. BURGYÁN

**Defective Interfering RNA-mediated resistance against
cymbidium ringspot tobramovirus in transgenic plants**

Agricultural Biotechnology Center, Gödöllő, Hungary

Defective interfering (DI) RNAs are deletion mutants of infectious viral genomes and are incapable of autonomous replication, but contain sequences necessary to be replicated. DI RNAs of plant viruses generally induce symptom attenuation, thus, plants expressing these small RNAs are expected to acquire resistance. A cymbidium ringspot virus (CyRSV) DI RNA coding sequence was cloned into a plant expression vector, between a cauliflower mosaic virus 35S promoter and the nopaline synthase termination signal region. Via *Agrobacterium tumefaciens* mediated leaf disc transformation procedure several lines of transgenic *N. benthamiana* plants were generated expressing the DI RNA sequences either in the positive (DI-13(+)) or in the negative (DI-13(-)) orientation relative to the cauliflower mosaic virus 35S promoter. Integration of DI RNA encoding sequences in the plant genome was verified using polymerase chain reaction amplification of DNA extracts purified from transgenic plants. Accumulation of RNA transcripts of the expected size was shown by Northern blot hybridization in RNA samples prepared from the transgenic DI-13(+) and DI-13(-) plants. Inoculation of the DI-13(+) and DI-13(-) transgenic plants with in vitro generated transcripts of the parenteral virus induced replication of DI RNA only in transgenic plants producing positive strand DI RNA transcripts. Sequence analysis showed that the amplified DI RNA was identical to that of the originally cloned one. Transgenic plants in which DI RNA accumulated exhibited only attenuated disease symptoms (e.g. the DI-13(+) plants) just like non transformed plants infected with inoculum containing DI RNA. On the contrary DI-13(-) plants showed apical necrosis and wilting of lower leaves as non transgenic plants. Inoculation of the transgenic plants with inoculum containing different concentrations of purified CyRSV lead to the same results in every occasion. These data indicate that novel type of virus-resistant plants, generically engineered with DI RNA sequences were successfully obtained.

T. DALMAY and J. BURGYÁN

**Rapid generation of dimeric defective interfering RNA
of cymbidium ringspot tomosvirus**

Agricultural Biotechnology Center, Gödöllő, Hungary

Cymbidium ringspot tomosvirus (CyRSV) supports the replication of defective interfering (DI) RNAs. CyRSV DI RNA was shown to consist of mosaic molecule made up entirely of noncontiguous regions of genomic RNA. Coinoculation of *Nicotiana clelandii* plants with in vitro transcripts of both genomic and DI RNAs resulted a rapid accumulation of a new DI like RNA species larger than the input synthetic DI RNA. This RNA was suggested to be a dimer form of input DI RNA inoculum, based on the size and the hybridization nature of the new RNA species. The supposed junctions of two monomeric units of CyRSV DI RNA were cloned and sequenced. Inoculation of plants with dimeric DI RNA transcripts led to a very efficient replication of dimer DI RNA and produced only a small amount of monomer form in the inoculated leaves of *N. clelandii*. However, in the upper non inoculated leaves unit length DI RNA were found almost exclusively as in monomer DI RNA inoculated plants. Our results showed that both forms of DI RNA can produce both monomer and dimer forms. DI RNAs having different sizes differ dramatically in the ability to generate dimeric forms. Coinoculation of synthetic genomic RNA with two different DI RNAs resulted in several types of dimer DI RNAs and sequence analysis showed recombination between the two different DI RNA molecules.

Z. HAVELDA, T. DALMAY and J. BURGYÁN

**Mutation analysis of a DI RNA of cymbidium ringspot
virus**

Agricultural Biotechnological Research Centre, Budapest, Hungary

Defective interfering (DI) RNAs of cymbidium ringspot virus (CyRSV) are formed from the genomic RNA of the virus by linear deletion. DI RNAs, being unable to replicate unaided, use a virus-coded polymerase enzyme for replication. The DI RNAs contain all the sequential and structural elements necessary in replication of the DI RNA and the viral genome. This work is aimed at identifying the sequences located in blocks and being indispensable in the replication of the DI RNA. A cDNA clone of one of the smallest DI molecules occurring naturally was incorporated into pUC-18 vector after the T7 phage promoter to create splitting loci for restriction enzyme by in vitro mutagenesis between sequence blocks. From the DI-22 block thus obtained, biologically active DI RNA can be transcribed with the help of the T7 RNA polymerase. When the Bal-31 exonuclease was used, DI-22 mutants of different lengths were produced, starting from natural and artificial restriction enzyme-

recognizing sites. The recombinant DI-22 plasmids were sequenced and *Nicotiana clevelandii* plants were infected with their in vitro transcripts, together with the transcript of CyRSV genomic RNA.

The RNA preparations extracted from infected plants were examined by Northern hybridization to show if recombinant DI molecules were capable of utilizing the viral polymerase enzyme in their replication.

The results thus obtained have enabled us to prepare the smallest DI RNA molecule capable of replicating. Thus, we can localize the regions which are located at the 3' and 5' ends of the virus or internally. Our results might considerably contribute to clarification of the mechanism of CyRSV replication.

L. PALKOVICS, J. BURGYÁN and E. BALÁZS

**Non-radioactive double sandwich hybridization test
for the detection of plum poxvirus**

Institute of Plant Sciences, Agricultural Biotechnology Center, Gödöllő, Hungary

One of the most devastating diseases of stone fruit trees is caused by plum pox virus (PPV). The complete nucleotide sequence of a Hungarian PPV strain (SK 68) was determined. The genomic RNA of PPV SK 68 is 9786 nucleotides in length. A clone containing the 2400 nts at 3' end was used for developing a new non-radioactive sandwich hybridization technique. The method based upon RNA-RNA hybridization. Plant material was homogenized in 0.5% SDS and added directly to the hybridization reaction, in which a pair of identifying probes was used. One of the probes was biotinylated capture RNA specific for PPV, the other RNA probe was synthesized from a plasmid bearing the adjacent sequence of the biotinylated probe and was labelled with digoxigenine. Both purified viral RNA and crude extracts from PPV infected plants were used as a target for sandwich hybridization. The hybridization reaction was carried out in streptavidin coated ELISA plate. After extensive washing the viral RNA was detected by the conventional color reaction using DIG-AP conjugate. The sensitivity of the test is 10^7 viral RNA molecules/sample. In comparative experiments we have shown that this non-radioactive detection system is more sensitive than conventional ELISA techniques and we were able to detect virus specific RNA in 50% of ELISA negative samples.

J. VARGA, A. VRIESEMA, Cs. VÁGVÖLGYI and F. KEVEI

Double-stranded RNA mycoviruses in *Aspergillus niger* strains

Department of Microbiology, A. József University, Szeged, Hungary, and Department of Genetics, Agricultural University, Wageningen, The Netherlands

Double-stranded RNAs (dsRNAs) have been found earlier in several fungal species. The presence of these molecules usually indicates the presence of virus-like particles (VLPs) in the cytoplasm of the strain examined. These VLPs were described to have several effects on the fungus, revealing phenotypic differences between the virus-containing and virus-free strains. No such phenotypic signs have been observed so far in *Aspergilli*. A search for dsRNAs and VLPs carried out in a large number of black *Aspergillus* strains representing several species. dsRNA bands were observed at high frequency (in 11% of natural isolates and in 13 out of 51 collection strains) on agarose gels after electrophoretic separation of nucleic acids. Electron microscopic examination of the strains revealed the presence of isometric VLPs in the cells. dsRNA bands with the same mobility, and VLPs with the same size were found in an *A. clavatus*, some *A. japonicus* and *A. niger* strains. No phenotypes related to the presence of these VLPs have been observed so far. Transfer experiments were also carried out between variously related *Aspergillus* strains.

I. MEDVECZKY, E. KUDRON, Zs. MÁTÉ, M. MARKOS-HALÁSZ,
J. MÉHESFALVI, E. MOCSÁRI, J. TANYI, M. TERÉNYI and V. KOVÁCS

**Isolation of Aujeszky's disease virus (SHV-1) in Hungary
between 1971–1991 from carnivores, ruminants and
rodents**

*Department of Epizootiology, Veterinary University, Budapest, Veterinary Institute of Szombathely
Miskolc, Békéscsaba, Kaposvár, Budapest and Debrecen, Hungary*

Between 1971–80 SHV-1 was isolated in Hungary with the exception of two counties (Tolna, Baranya) from ruminants and carnivores. Between 1971–91 most virus isolations were reported from 113 dogs and from 101 cats. Among ruminants, the virus was isolated from 11 sheep and 7 cattle. Most of the virus isolations occurred in counties situated on the Plain (counties Pest, Csongrád, Békés, Hajdú). In Baranya county (Trans Danubian area) between 1972 and 1990 SHV-1 was isolated only from pigs. The number of virus isolations from carnivores and ruminants in yearly distribution showed similar tendency to the ones from pigs.

Most of the isolations were reported in 1975, 1981, 1986 and the less of them in 1973 and 1979. Changes in the isolation frequency showed a similar seasonal character (early spring and late autumn peak), like the isolations from pigs, with the difference that there was a significant increase in August. Successful isolations were carried out in

1981 and 1982 from mice and in 1983, 1990 from fitches. The double sandwich hybridization test for the virus SHV-1 was isolated from rabbits in Vas county in 1981 and in Békés county in 1988, SHV-1 was isolated from foxes in 1980, 1983, 1984, 1987 and 1990.

I. MEDVECKY, E. KUDRON, Zs. MÁTÉ, M. MARKOS-HALÁSZ,
J. MÉHESFALVI, E. MOCSÁRI, J. TANYI, M. TERÉNYI and V. KOVÁCS

**The spread of Aujeszky's disease in Hungary on the basis
of the number of virus isolations from pigs
between 1971–1991**

*Department of Epizootiology, Veterinary University, Budapest, and Veterinary Institute of Szombathely,
Miskolc, Békéscsaba, Kaposvár, Budapest and Debrecen, Hungary*

In Hungary there were virus diagnostic departments in three Veterinary Institutes (Budapest, Debrecen, Miskolc) between 1971–74, which enabled the continuous virus isolation in 11 counties. From 1975 onward virus isolation was extended by the network of the Veterinary Institutes to all 19 counties of Hungary. Aujeszky's disease virus (SHV-1) was isolated from pigs in 2938 cases between 1971–91. The yearly distribution of the virus isolations showed a 4-year periodicity. Most of the isolations occurred in 1977, 1979 and 1981. The number of virus isolations, in case of similar herd density, was higher in those counties where breeding stocks with higher number of animals were predominating (e.g. Hajdú county 563, Csongrád county 72). The number of virus isolations was lower even in case of higher herd density, if the herds with fewer number animals predominated in the county (e.g. Győr county 50, Tolna county 81). A close connection was observed between the herd density, the number of sows and the number of virus isolations. The distribution of virus isolations showed a seasonal character. Most isolations happened in January–March, and/or November–December, the less in the summer months.

E. DANCZIG and G. BULLA

**Serological estimation of bovine leucosis in counties
Békés and Csongrád**

*Animal Health Institute, Békéscsaba, and Csongrád County Animal Health and Food Control Station,
Szeged, Hungary*

The cattle stock of Hungary was screened for leucosis late in 1992 as prescribed by the EC. In the two counties for which the Animal Health Institute, Békéscsaba is competent the following results were obtained. In Békés county, where 8883 animals were screened, 324 out of 3412 farms (9.4%) proved positive. In Csongrád county, where blood samples from 8779 animals were tested, only 102 farms out of 3750

(2.7%) were positive. With regard to the low positive rate for Csongrád county the individual cattle kept in small yards were re-examined in that country. The double-negative farms may be considered free from leucosis. The above data, being informative, may serve as an example of elimination in due time at county level and may represent the first steps of country-wide eradication of the disease.

SZ. KISS

**Role of echoviruses in the etiology
of acute polyradiculoneuritis**

Laboratory of Virology, Institute of Public Health and Medical Research, Cluj, Romania

Acute polyradiculoneuritis, a severe impairment of the nervous system caused by echoviruses has been considered to be of outstanding importance among the clinical polymorphism of echovirus infections. Virus isolation experiments have been performed from different diagnostic samples (nasopharyngeal secretions, throat swabs, stool samples, cerebrospinal fluid and blood clots) using the HeLa cell line in order to elucidate the role of echovirus infections in the etiology of acute polyradiculoneuritis cases. Virus neutralization tests were also performed using the available echovirus serotypes 1, 3, 4, 5, 8, 10, and 17. Two hundred-fifty-six serum samples and 441 samples for virus isolation were examined taken from patients of 1 to 62 years of age, suffering from different neurological diseases. One single blood sample from each patients was tested in the virus neutralization test. The virus neutralizing titre of 1:128 has been considered to be of diagnostic value. It has been concluded that in the majority of polyradiculoneuritis cases (84%) the probability of a recent echovirus infection can be taken into consideration. Age groups of 5 to 9 years (30.7%) and 16 to 24 years (23%) were found to be mainly affected. The seasonal peak of the incidence was observed in the summer and autumn months (June to September). The results indicate an intensive circulation of echoviruses in 1992. Serotypes 3, 10, and 17 were the most prevalent in 1992.

I. MIHÁLY, J. BUDAI, A. GERŐ and E. KUKÁN

**Cases of acute parotitis following previous
morbilli/mumps/rubella vaccination**

St. László Hospital for Infectious Diseases, Budapest, Hungary

Ten children who had previously received Morbilli/Mumps/Rubella (MMR) vaccine developed parotid swelling diagnosed as acute parotitis 11 days to 5 months after vaccination. Blood samples from each of the patients were tested for the following virological parameters: Mumpsvirus, Parainfluenzaviruses (PIV) types 1, 2,

3, Respiratory Syntytial Virus (RSV), Epstein Barr Virus Capsid Antigen (EBVCA) IgM, IgA, IgG; Epstein-Barr Virus Early Antigen (EBV EA) IgG immunofluorescent test (IFT); Adenovirus, Influenza A, B Complement Fixation (CF) test. Some of the sera were examined for CMV IgM, IgG ELISA (Organon Teknika) and Human Parvovirus B19 IgM, IgG recombinant ELISA (Bender), too. The etiological role of PIV-2 and PIV-1 was confirmed in six and two patients, respectively. Besides the PIV-2 in two patients and PIV-1 in two other patients mumpsvirus IgM antibody in a very low dilution and mumps IgG antibody in a dilution 1:4 to 1:640 were found. In only one of the ten children could the diagnosis of mumps be confirmed. This case was interpreted as a clinical reaction of mumps vaccination. The ethiology was unknown in the remaining one patient. The results show the importance both of the broad spectrum precise serological studies and of the skilful interpretation in the exact diagnosis of the acute parotitis to identify parotitis either as a consequence of the mumps vaccine or vaccine failure. The correct diagnosis of acute parotitis can influence the booster mumps vaccination practice, too.

I. MIHÁLY, E. KUKÁN, M. GELLÉRT, A. GERŐ and F. MÁNDOKY

**Acute parotitis associated with viruses other
than mumpsvirus**

St. László Hospital for Infectious Diseases, Budapest, Hungary

Blood samples of 204 acute parotitis patients in a fifteen month period (1991–1992) were systematically examined for IgM, IgA, IgG antibodies of mumps and parainfluenzavirus 1, 2, 3, (PIV) by immunofluorescence test (IFT) and, in special cases, several other virological examinations were done. The etiological role of mumps, parainfluenzavirus 1, 2, 3, and one of the other viruses was confirmed in 62.0%, 11.7%, 5.4%, 2.0% and 7.4% of the cases, respectively. The ethiology remained unknown in 11.8%. There were clinical symptoms of meningitis or orchitis in some and lymphadenopathia in several patients with parainfluenzavirus parotitis. It may be concluded that one or other serotype of the parainfluenzaviruses is the second most frequent etiological agent of parotitis next to mumpsvirus. Respiratory Syncytial Virus (RSV) also plays an etiological role in parotitis. This observation should be confirmed in the future by some other kinds of virological tool. There are difficulties and pitfalls of the virological serology in the infections caused by paramyxoviruses (PMV).

M. BENKŐ and B. HARRACH

**Identification of the proteinase gene of bovine adenovirus
type 4**

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

The proteinase gene of bovine adenovirus (BAV) type 4 has been identified by detailed physical mapping, subcloning and sequencing the cloned viral DNA. The size (600 bp) and the location (around map unit 58) of the gene was corresponding to that found in other animal and human adenoviruses (HAVs) studied so far. It can be concluded, that in spite of the significantly smaller genome size, the genome organization of the subgroup 2 BAVs is similar to that of HAVs. The DNA sequence of the proteinase gene showed a special codon usage resulting in an AT content of over 66%. Such high AT content can be observed in the proteinase gene sequence of BAV-7 (another member of subgroup 2 BAVs) only. If this high AT content observed in other BAV-4 sequence data as well is present in the entire genome, then, very likely, this is that molecular biological property, which makes subgroup 2 BAVs so distinct (no cross reaction in DNA hybridization experiments with any other Mastadenovirus strains).

Gy. PREMECZ, A. MARKOVITS, G. VAJDA and I. FÖLDES

**"Mitogenic-stimuli" dose-dependently activate or inhibit
vesicular stomatitis virus (VSV) RNA-synthesis**

Microbiological Research Group, B. Johan National Institute of Hygiene, Budapest, Hungary

The effects of different mitogenic stimuli on VSV replication were studied by measuring ³H-uridine incorporation into the viral RNA. Before (or after) infection with a standard test dose of VSV human amnion cells (UAC) were treated with substances epidermal growth factor (EGF), concanavalin A (Con A), phytohaemagglutinin (PHA), lipopolysaccharide (LPS), interferon- α (IFN- α), polyinosinic-polycytidylic acid (poly(I):(C)), VSV, UV-inactivated VSV (VSV_{uv}) and glycoprotein isolated from VSV known to induce a mitogenic response. At low doses all these substances stimulated the replication of VSV, while at high concentrations a significant inhibitory effect was measured. These results suggest common (or at least overlapping) mechanisms in signal transduction of mitogenic stimuli and in inducing antiviral state of the cells.

J. MINÁROVITS, L. F. HU, S. IMAI, Y. HARABUCHI, A. KATURA,
S. MINÁROVITS-KORMUTA, T. OSATO and G. KLEIN

**Clonality, expression and methylation patterns of the
Epstein-Barr virus genomes in lethal midline granulomas
(LMGs)**

*Microbiological Research Group, B. Johan National Institute of Hygiene, Budapest, Hungary,
Department of Tumour Biology, Stockholm, Sweden, and Department of Virology, Cancer Institute,
Hokkaido University, Sapporo, Japan*

We analysed the terminal repeats (TR) of Epstein-Barr virus (EBV) in DNAs isolated from six lethal midline granuloma (LMG) biopsies. A single fused terminal fragment could be detected in each case, indicating that these angiocentric peripheral T-cell lymphomas represent clonal proliferations of cells infected with EBV on a single occasion. Using the method of RT-PCR, we detected EBNA 1 and LMP 1, but not EBNA 2 messages in LMG biopsy RNAs. The splicing pattern of the EBNA 1 message was consistent with the usage of a promoter localized in the BamHI F fragment (F promoter). The BamHI W repeats and LMP coding sequences were highly methylated in all cases. In contrast, the LMP regulatory sequences (LRS) were found to be hypomethylated or partially methylated, like in LMP expressing nasopharyngeal carcinomas.

Gy. VERESS, J. KÓNYA, T. CSIKY-MÉSZÁROS, J. CZEGLÉDY and L. GERGELY

**Human papillomavirus (HPV) DNA and anti-HPV
secretory IgA antibodies in cytologically normal cervical
specimens**

*Institute of Microbiology, University Medical School, Debrecen, and District Gynecologic Outpatient
Clinic, Debrecen, Hungary*

Cervical specimens collected from 163 cytologically normal women were screened for the presence of human papillomavirus (HPV) DNA and anti-HPV secretory IgA antibodies. HPV DNA was detected by a general primer mediated polymerase chain reaction (PCR), which amplifies a conserved region from the L1 ORF of genital HPVs. The PCR products were typed by restriction enzyme digestion. A total of 35 samples (21.5%) were positive for HPV DNA. HPV DNA positivity was significantly higher among women under 25 years of age (34.8%) than among the older patients (12.4%; $p < 0.001$). An enzyme-linked immunosorbent assay (ELISA) using synthetic peptide antigens was performed to detect local secretory IgA antibodies against different HPV specific antigens. Thirty-four secretions (20.9%) were found to react with at least one of the oligopeptides. Anti-HPV IgA positivity was the highest among women aged 25–32 years, and it was significantly lower in both the younger and the older age groups ($p < 0.05$). Correlation between HPV DNA and anti-HPV IgA

detection was positive but rather weak ($p < 0.05$); the latter can be explained by the fluctuating course of latent genital HPV infections.

J. KÓNYA, Gy. VERESS, L. HERNÁDI, J. CZEGLÉDY and L. GERGELY

**The effect of the high-risk human papillomavirus types
on the development of cervical cancer**

*Department of Microbiology, Department of Obstetrics and Gynecology, University Medical School,
Debrecen, Hungary*

High-risk human papillomavirus types (HPV 16, 18 etc.) are strongly associated with carcinoma of cervix uteri. The infection generally appears in latent and subclinical forms. Though the viral prevalence has the peak in the young adult population (age 20–25), the majority of cervical cancer is detected at about two decades later, above 40. On the other hand, increasing number of cases show younger age at onset with worse prognosis and rapid development. In the latter group, the role of HPV 18 has been suggested because of its higher efficiency for transformation in vitro compared to HPV 16. The viral DNA in clinical biopsies is detected by a general primer mediated polymerase chain reaction (PCR). Amplified products are typed by restriction enzymes. In the group under 35, the preliminary study revealed HPV 16 and HPV 18 in 5 and 4 cases of 9, respectively. In the older group 15 of 19 samples were positive for HPV 16 and 3 of 9 HPV 18. The study is continued in order to assess the role of HPV 18.

Zs. GYULAI, B. PRÁGAI, I. MARCZINOVITS and I. BÉLÁDI

**Immunologic analysis of recombinant vaccinia virus
expressed HIV-1 *env* proteins by sera from AIDS patients
without vaccinia virus specific immunoglobulins**

Institute of Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary

We constructed recombinant HIV-1 vaccinia viruses which carry a part of the *pol* (pHIV1-BH10: 2101–2882 nt) and the *env* genes (5737–7146 nt, 6662–8624 nt). For heterologous gene expression, BSC-40 and SW 480 cell lines were infected with recombinant isolates and the gene products expressed were determined by Western blot with the ELISA positive sera from AIDS patients who were not immunized with vaccinia virus. Out of three recombinants, only one (6662–8624 nt) expressed proteins which were detectable by Western blot and the size of the products was identical with that of the cloned and processed HIV-1 gene *env* coded proteins.

Á. GYURIS, G. VAJDA, J. SEGESDI, I. FÖLDES and Zs. JELENIK

Demonstration of HIV activity in the offspring of a mother suffering in AIDS. Case report

Microbiological Research Group, B. Johan National Institute of Hygiene, Budapest, and St. László Hospital for Infectious Disease, Budapest, Hungary

According to literary data offsprings of HIV-infected mothers become HIV carriers in 20–40% by infection during pregnancy, delivery or by breast feeding. In the first 18 months HIV infection cannot be diagnosed by serological examination, since the origin of antibodies is dubious. At present four methods are available to prove infection: antigen assay, polymerase chain reaction (PCR), in vitro antibody production (IVAP) and virus culture assay (VCA). F. N., male, born in July 1992 by an AIDS-diseased mother was studied by IVAP and VCA. Lymphocytes isolated from the heparinized blood of the infant were cultured at two occasions (January and April, 1993) in the presence of PHA and IL-2, or Epstein-Barr virus (EBV), respectively. Coculturing the infant's lymphocytes with PHA-stimulated normal donor lymphocytes, a cytopathic effect could be observed at the 7th day of cultivation. Using a known HIV positive serum, a positive immunofluorescence could be observed simultaneously. In supernatant of EBV-stimulated lymphocytes no HIV-specific antibodies could be detected by ELISA: thus IVAP gave a negative result. Similar results were obtained in April 1993. We conclude that by combining different methods detection of HIV infection of infants may be possible even in laboratories having no facilities for PCR.

B. SZABÓ, C. LOCARDI, E. LoPRESTI, F. BELARDELLI and A. BENEDETTO

TNF-alpha increases the sensitivity of HIV-infected cells to monocytotoxic antibodies

Department of Microbiology, University Medical School, Debrecen, Hungary, Department of Virology, Central Health Institute, and Centre of Virology, San Camillo Hospital, Rome, Italy

One third of serum samples of 40 HIV-seropositive drug addicts proved to be cytotoxic against HIV-infected U937 monocytic cells in the presence of complement. The pretreatment of target cells with TNF-alpha increased the detectability (up to 60%) and the percentage (from 16 ± 4 to 70 ± 5) of serum cytotoxicity. This activity of sera could be reduced by treatment of sera with recombinant HIV pg120 antigen. This reduction was dose-dependent in the majority of cases. Morphological studies indicated that the density of target antigens was elevated in the cell membrane of TNF-treated U937 cells. These results suggest that endogenous TNF-alpha can be a "protective factor" since it can render persistently infected cells highly sensitive to serum cytotoxicity, as a result of increased expression of HIV pg120, as revealed by Western-blot analyses of target cell-membrane extracts.

I. ROSZTÓCZY, B. K. RAJ and P. M. PITHA

Investigation of the mechanism of priming, IFN promoter sequence requirements and IRF-1 mRNA expression

*Institute of Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary,
and The Johns Hopkins Medical University, Oncology Center, Baltimore, MD, USA*

A comparison of the inducibility and the enhanced expression by interferon (IFN) pretreatment of various murine IFN-(MuIFN)-alpha 4 promoter deletion mutant chloramphenicol acetyltransferase (CAT) constructs indicated that priming did not alter the IFN gene promoter structural requirements for inducibility. Primed cells incubated and subcultured in IFN-free medium retained the primed state for at least 48 h. In contrast to priming, the induction of IFN regulatory factor 1 (IRF-1) mRNA synthesis by IFN pretreatment had only a short duration. Cells transiently transfected with MuIFN-alpha 4 promoter CAT gene hybrid construct expressed more CAT activity if they were cotransfected with an IRF-1 expression plasmid. However, the induction of the CAT gene by IRF-1 was further increased by priming. The enhancing effect of priming on CAT expression could also be demonstrated in cells induced with Sendai virus after transfection.

K. MEGYERI, S. SZABÓ, B. SZENTKERESZTY and I. ROSZTÓCZY

Interferon induced modulation of T lymphocyte apoptosis

Institute of Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary

Programmed cell death (PCD) or apoptosis was induced with different dilutions of anti-CD3 monoclonal antibody in peripheral T lymphocytes obtained from healthy donors. The ratio of apoptotic cells was determined by multiparameter flow cytometry and the characteristic DNA fragmentation was detected by agarose gel electrophoresis. The kinetics of apoptosis was followed by examination of the DNA extracted at 10, 24, 48 and 72 h after induction. Initial chromatin damage occurred at 10 h after induction, which was followed by a pronounced DNA fragmentation at 24 h after anti-CD3 antibody treatment. Samples obtained at later intervals indicated less or no DNA damage, suggesting that a DNA repair mechanism had taken place in a certain portion of the cells treated with the monoclonal antibody. Interferon was applied in concentrations 1, 200 and 1000 IU/ml to investigate its possible effect on PCD. Apoptosis induced by high anti-CD3 concentrations was inhibited by interferon, while DNA fragmentation induced by low doses of the anti-CD3 antibody was augmented by interferon. These observations indicate that interferon differentially regulates T lymphocyte apoptosis induced by anti-CD 3 monoclonal antibody.

I. MUCSI, I. BÉLÁDI, J. MOLNÁR, K. BURIÁN, T. KURIHARA
and N. MOTOHASHI

**Effect of phenothiazine and benzo(a)phenothiazine
derivatives on the multiplication of herpes simplex virus
in cell culture**

*Institute of Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary, Department of
Organic Chemistry, Technical Sciences Josai University, Saitama, Japan, and Department of Medicinal
Chemistry, Meiji College of Pharmacy, Tokyo, Japan*

The effects of phenothiazine and benzo(a)phenothiazine derivatives on the multiplication of herpes simplex virus type 2 (HSV-2) were studied in Vero cells by the "yield" reduction test. The compounds depending on the concentration had virostatic effects, however, generally the benzo(a)phenothiazines were more effective than the phenothiazine derivatives. A relationship could be suggested between their antiviral activity and chemical structures. Further studies are needed to determine the mechanism of antiviral activity of phenothiazine derivatives.

Zs. RUZSICS, T. TAKÁCS, A. KISS, É. ÁDÁM and I. NÁSZ

**Isolation and amplification of antiviral monoclonal
antibody genes**

Institute of Microbiology, Semmelweis University Medical School, Budapest, Hungary

mRNAs transcribed from the immunoglobulin genes of mouse hybridoma cells were isolated. This cell line was developed against the crystallized adenovirus type 1 hexon, and the antibody specificity was characterized previously. The purification of mRNA was performed by oligo-dT-cellulose affinity chromatography. The first-strand cDNA was generated from the mRNA by reverse transcription using random hexadeoxyribonucleotides. The synthesized first-strand antibody cDNA was used as a template for PCR amplification to generate suitable quantities of the antibody heavy and light chains encoding DNA for cloning. The amplification was carried out in two separated reactions with primer pairs which bind specifically to the conserved regions of the antibody genes. The amplified heavy (about 340 bp) and light (about 325 bp) chain DNA fragments were purified by agarose gel electrophoresis. Our next aim is to link the two chains, to clone the product into the pCANTAB5 phagemid vector and to express phage-display recombinant antibodies by M13K07 helper phage. This is a special way of cloning and expression of antibody genes from different kind of sources in *Escherichia coli*, based on a phage-display system in which fragments of antibodies are expressed as fusion proteins with the phage g3p proteins and displayed on the phage surface.

I. BÉLÁDI, K. SZALAY and M. BAKAY

**Delayed-type hypersensitivity in mice induced
by human adenoviruses**

Institute of Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary

Density gradient purified human adenovirus types 1, 3, 5 and 6 induce delayed-type hypersensitivity in mice inoculated subcutaneously into the hind footpad. To elicit delayed-type hypersensitivity virus was inoculated into the left footpad followed by an inoculation of the virus in the right footpad 7 days later. Large amount of virus (10^8 – 10^{10} TCID₅₀) was necessary for both the sensitization and the elicitation. Delayed-type hypersensitivity was pronounced when mice were treated with cyclophosphamide just before the inoculation of the sensitizing virus. The same virus dose injected intraperitoneally did not induce delayed-type hypersensitivity but resulted in a decreased humoral immune response to sheep red blood cells inoculated four days after the virus injection. Cross-reactivity was observed between types investigated, thus the common hexon antigen of adenovirus plays a role in the induction of delayed-type hypersensitivity.

A. KÁTAI, M. BENKŐ, E. SZÖLLŐSY, G. SCHWEIGER, E. SCHREIER
and M. ZIMÁNYI

**Intratypic characterization of type 2 polioviruses (PV 2)
by traditional and molecularbiological methods**

*Csongrád County Institute, National Public Health Service, Szeged, Hungary, and Robert Koch Institute,
Federal Health Service, Berlin, Germany*

The differentiation of vaccine derived and wild strains has significance in the control of poliovirus isolates. The traditional methods used by us for the intratypic characterization were the genetic markers as e.g. the rct/40 and Al(OH)₃ gel elution marker, but according to published data these properties are subject to change in the course of passages in the population. The newly developed molecular method using genomic amplification (PCR) followed by selective restriction enzyme digestion made it possible to determine the differences between Sabin vaccine and wild-type PV strains. This method is based on the conserved and variable components of the 5'-noncoding region of the genome. Five restriction enzymes were selected to allow an unambiguous identification of the investigated strains on the basis of the restriction fragment length polymorphism (RFLP analysis). The above-mentioned methods were used to differentiate between the PV 2 Sabin (P 712 Ch 2ab strain) and PV 2 5148-II recommended by the WHO as wild reference strain and the MEF 1. The strains could be differentiated exactly, and these methods were used to characterize further 16 PV 2 strains isolated in the virology laboratory of the Csongrád County Institute of the

National Public Health Service in 1992. On the basis of our examinations, 4 of the investigated strains were identical to Sabin 2 in the analyzed region.

Gy. SZŰCS, D. O. MATSON, M. UJ, E. KUKÁN, I. MIHÁLY, Zs. JELENIK
and M. K. ESTES

Prevalence of rotavirus G serotypes in two regions of Hungary

Baranya County Institute, National Public Health Service, Pécs, Hungary, St. László Hospital for Infectious Diseases, Budapest, Hungary and Division of Molecular Virology, Baylor College of Medicine, Houston, Texas, USA

The first longitudinal serotyping (G typing) data of group A rotaviruses in Hungary are reported. Enzyme immunoassay incorporating neutralizing monoclonal antibodies specific for the VP7 protein of serotypes 1, 2, 3, and 4 were used to determine the antigenic variation of group A rotaviruses in two collections of stool specimens. Of a total of 11 031 samples collected from 1984–1992 in Baranya County and from 1988–1992 from the St. László Hospital for Infectious Diseases in Budapest, 28% and 29% contained rotaviruses, respectively. Ninety-two percent of the 1285 tested samples were typed as G1 (81%), G2 (4%), G3 (1%), and G4 (5%), and mixed type (1%). Serotype G1 was the predominant type during the entire investigated period, with one exception in Baranya County, where a predominance of type G4 was observed in the 1988/1989 rotavirus season. With two year periodicity, serotype G2 circulated in both regions, and rotaviruses of type G3 were not revealed in the last two seasons. These first serotyping data from Hungary are compared to those published previously of other European countries (UK, Finland, Italy, and Sweden).

I. SISKÁ, I. MEZEY, M. TAKÁCS and E. HÜTTER

Prevalence of the human herpesvirus type 6 in Hungary

B. Johan National Institute of Hygiene, Budapest, Hungary

A new member of the herpesvirus group, discovered in 1986, is the serotype 6 (HHV6), which replicates in human T-cells, and has been shown to cause the juvenile human disease exanthema subitum. In spite of the fact that the growing proportion of the Hungarian population has been vaccinated against rubella and measles, exanthematous diseases occur rather frequently. It seemed to be necessary to test the role of HHV6 as the causative agent of these infections. Part of the blood samples have been obtained in connection with the Program of optimal Family Planning, from clinically healthy pregnant women between 20 to 40 years of age, in order to test their rubella antibodies. The majority (72.7%) of them were found to possess HHV6-specific IgG. The virus carrier state in Hungary was shown to be very frequent, since the IF

reagent used will give positive results only in the case of recent reactivations (protocol of the manufacturer). The seropositivity rates are between 18 to 88% according to the published data. The results obtained using selected blood samples, which had been pretested for the absence of rubella, measles, human parvovirus B-19, Epstein-Barr virus, and adenovirus specific antibodies indicated that 41 of 128 such patients suffering from exanthematous diseases (i.e. 1:3 of them) HHV6 infection could be verified using the detection of specific IgM type antibodies with indirect immunofluorescence test. Characteristic clinical symptoms of the illnesses caused by HHV6 were fever, exanthemes of different morphological appearance, and progressive for several days, enlargement of lymph nodes, and upper respiratory symptoms. The majority of 3 years old children were found already to be seropositives. Eight of 57 children younger than 3 years of age were seronegative, supporting the published data, that the babies are infected during the lactation period. The preliminary Hungarian data also suggest that 0.9 part of the children are infected with HHV6 in the first two years of their life. Nevertheless, the specific IgM tests for HHV6 antibodies revealed that 18 of 41 verified primary infections of the selected children suffering from exanthematous diseases acquired the primary HHV6 infection later than the 12th month of the life. These data provide indirect evidence for the facts that the epidemiological significance of childrens' communities are important, and that at least two HHV6 serotypes might circulate in the country.

I. MEDVECZKY and J. SZILÁGYI

Summary of the Hungarian literature of Aujeszky's disease

Department of Epizootiology, Veterinary University, Budapest, and Department of Microbiology, A. József University, Szeged, Hungary

Results of the experiments concerning Aujeszky's disease have been summarized on the basis of the Hungarian scientific literature from 1902 to 1992. The processing was carried out on the basis of the data of the Veterinarius, Állatorvosi Lapok (Veterinary Papers) and Magyar Állatorvosok Lapja (Journal of the Hungarian Veterinarians) in Hungarian as well as the Acta Microbiologica Academiae Scientiarum Hungaricae and Acta Veterinaria Academiae Scientiarum Hungaricae (the later two in English).

In the aforementioned journals, concerning the disease, 127 authors published 125 articles. Most of the publications dealt with diagnostics. Most of the articles were published in 1990 (11 articles) as well as in 1962, 1965, 1978 (8-8 articles). It was an interesting observation that there were no publications between 1915-23, 1928-32, 1943-49 and 1970-73. The authors were divided into groups according to the number of publications and the field of science. The most significant publications among the several outstanding ones, were the first description of the disease (A. Aujeszky: Veterinarius 1902) and the development of the first vaccine (A. Bartha: Magyar Állatorvosok Lapja 1961).

I. MEDVECZKY, F. KOVÁCS Sz., A. KÜKEDI and O. GYULASI

**Aujeszký's disease virus (SHV-1) challenge dose titration
on 7 days old piglets**

*Department of Epizootiology, Department of Chemistry, Veterinary University, Budapest, Hungary, and
Boehringer Ingelheim Vetmedica GmbH., Ingelheim am Rhein, Germany*

Since older pigs seems to be more resistant to Aujeszký's virus (SHV-1) infection than younger (e.g. suckling or weaning) ones the optimal challenge dose had to be determined for each age group separately. The study aimed to determine the optimal intranasal (i.n.) virulent virus challenge dose of 7-day old piglets for the immunogenicity study (passive immunization) required in the European Pharmacopoeia. The litters of 3 pregnant sows were used, each representing an experimental group. Seven-day old piglets were infected i.n. with the 10^1 , 10^2 and 10^3 PFU/ml nostril virulent virus. Rectal temperature and body weight of the piglets were measured and blood, nasal swabs were collected from the piglets. During the challenge experiment in 7-day old piglets half of the piglets died on the 4th and 5th post challenge day (PCD) due to the intranasal infection with 10^1 PFU/ml/nostril challenge dose of SHV-1. The rest of the litter was infected by the higher value virus shed by piglets infected on day of challenge, and perished between 9–12 PCD. The 10^2 and 10^3 titre challenge dose caused 100% mortality between the 4th and 5th PCD. In the immunization experiments the 10^2 and 10^3 minimal challenge dose can be suggested for intranasal infection of seven-day old piglets. The study was conducted according to GCP (good clinical practice) requirements.

I. MEDVECZKY, F. KOVÁCS Sz., A. KÜKEDI and O. GYULASI

Challenge dose titration study of SHV-1 in fattening pigs

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Boehringer Ingelheim Vetmedica GmbH., Ingelheim am Rhein Germany*

The study aimed to determine the optimal intranasal (i.n.) virulent virus challenge dose of fattening pigs for the immunogenicity study required in the European Pharmacopoeia. Altogether 15 fattening pigs (80–100 kg) were used in the study in 3 experimental groups infected i.n. with 10^4 , 10^5 and 10^6 PFU/ml/nostril. The body temperatures were measured every day, the clinical data were recorded twice a day and the weight data were recorded every other day plus on day 7 after challenge. On the basis of the statistical evaluation of the results there was no significant difference between the groups challenged with 10^4 , 10^5 and 10^6 PFU/ml/nostril challenge dose. Considering the values of the body weight changes all the three infected groups met the challenge values required by the European Pharmacopoeia. In the immunization experiments the given test conditions decide, which challenge dose (10^4 , 10^5 and 10^6

PFU/ml/nostril) will be used. The study was conducted according to GCP (good clinical practice).

I. MEDVECZKY and B. LOMNICZI

**Examination of pig-pathogenicity of SHV-1 strains
isolated from sub-clinical field cases**

*Department of Epizootiology, Veterinary University, Budapest, and Veterinary Medical Research
Institute, Hungarian Academy of Sciences, Budapest, Hungary*

The pig pathogenicity of 5 strains (S, C6, C11, RK, M7) was examined on 8-week old piglets, originating from an Aujeszky's disease free farm. The S strain was isolated from semen of boar 4 months after vaccination with Bartha K-61 strain. C6 and C11 were plaque clones of the same nasal swab collected from a 3-week-old pig with mild symptoms of Aujeszky's disease. RK and M7 were isolated by cocultivation of trigeminal ganglia from old sows infected sub-clinically by Aujeszky's disease. Groups of 5 piglets were infected with 10^4 PFU/ml concentration of the given strain, intranasally. After the challenge the body temperature of the piglets was measured every day and the clinical data were recorded twice a day. Weight data were recorded every other day plus on day 7 after challenge. The titre of virus in nasal secretion was measured and given in PFU during 11 post challenge days (PCD) and on the 14th PCD. The strains S and C6 exhibited pig-pathogenicity values characteristic of strain K-61. Strains M7 and RK as well as C11 showed values characteristic of the virulent strains. The results of the animal experiments were supported by the RFP analysis of viral genomes as well as the detection of Ig antibody levels of pigs sera (L.S.Christensen et al.: Archives of Virology 1992. 124: 225-234).

IMMUNOLOGY

L. ZALAY and N. LENDVAI

New aspects of autovaccine therapy

St. Margit Hospital Budapest and Human Co. Ltd., Gödöllő, Hungary

Application of autovaccine has to be taken into consideration in the case of chronic and returning bacterial infections. The inflamed barrier formed frequently in the case of chronic bacterial processes decreases the effectiveness of antibiotics, and partly inhibits the activity of macrophages and other cells. Selection of the strains necessary for the production of autovaccine has to be carried out very carefully. A good result can be achieved only if the suitable microbe is isolated from the mixed

cultures. In the case of *Escherichia coli* an agglutination test is suggested in order to help the selection. Considering that autovaccines do not contain adjuvant agent, the treatment has to be carried out by intracutaneous injections during several weeks.

Zs. SZÉNÁSI, Zs. OZSVÁR, M. JESZENSZKY, M. KÁLMÁN and M. ZIMÁNYI

**Diagnostic value of anti-P30 specific IgA antibodies
in the determination of the stage of toxoplasmosis**

*Csongrád County Institute, National Public Health Service, Szeged, and Municipal Hospital, Szeged,
Hungary*

Primary maternal infection with *Toxoplasma gondii* during pregnancy may cause congenital toxoplasmosis in the fetus. Since the acquired toxoplasmosis in immunocompetent patients is generally asymptomatic, it is rarely detected in the mother. Thus, the offspring can escape the infection only by prospective screening and treatment of the infection of the mother. Since 1987, we have been routinely screening all the pregnant women in Szeged for toxoplasma antibodies. They are tested in the first 16 weeks of the gestation. The seronegatives are retested in every second month throughout the pregnancy to discover the possible seroconversion and, in case of it, the mother will be treated until delivery and a special follow-up will be arranged for the child. The protocol of our program has been approved by the Human Investigation Review Board. All sera are tested by complement fixation test (CFT) and, occasionally by ELISA IgG and/or IgM. Since IgM titres might persist for years in adult and might not be detectable in infected newborns, it was of interest to find additional markers of acute infection. Recently, it was shown that IgA antibodies do not persist as long as IgM might and do not cross the placenta and are found more frequently than anti-P30 IgM in infected fetuses and newborns. It was also found that the P30 protein, a major surface antigen of tachyzoites, provokes an immune response in the early stage of infection. Thus, in 1991 we introduced the determination of both specific IgA and IgM antibodies to P30. In a retrospective analysis, anti-P30 IgA antibodies were found in the sera of all pregnant women with seroconversion indicating acute toxoplasmosis. In the chronic phase of toxoplasmosis when IgG continued to rise and IgM antibodies also persisted ("residual IgM"), IgA was not detected. In conclusion, if specific IgM and IgA antibodies to P30 are simultaneously detected in the serum of a screened pregnant woman, the patient should be considered as having acute toxoplasmosis.

J. ZALA, Zs. HORVÁTH, A. SZÁNTÓ, É. KUYALEK, P. OSVÁTH,
E. K. NOVÁK and T. NAGY

Examination of fungal allergens in the air of Budapest
I. Theoretical possibilities

*B. Johan National Institute of Hygiene, Jahn Ferenc Hospital, and Children's Sanatorium, Szabadsághegy
Budapest, Hungary*

The allergic diseases caused by pollens are well known, and the environmental examinations connected with them have been covered widely. The allergies in the respiratory system caused by fungi in the air, however, are rarely known, but their occurrence and importance is increasing. Mycology methods are not yet standardized, nor are the critical levels fixed. Studying the possibilities of the standardization, we have focused our attention on the usefulness of the Burkard-trap used in pollen examinations and tested it for mycological purposes. From the analyses of about 100 Burkard-trap samples we came to the following conclusions: only some particles – mostly vegetative germinating forms – of ubiquitous fungi occur in the air. These fragments are the ones absorbed on the Burkard-trap layer. There can be conidia, spores, or maybe fragments of vegetative forms on it. Several representatives form typical identifiable conidia (*Alternaria*, *Helminthosporium*, *Ulocladium*, etc.). These strains can be identified on Burkard-slide under microscope. The Burkard-trap can be used for the identification of the species in the group mentioned above. Nevertheless, the major part of the fungal species form no conidia having identifiable morphological status. There is no diagnostical importance of the globose, ovoid conidia, however, the amount of them can be evaluated. For the clarification of the aetiology of fungal allergies the determination of the status of fungi can be necessary, as a whole fungi-flora (fungia), both qualitatively and quantitatively. In this case, new procedures should be introduced based on culture. Only the ones applied to the volumetric assay are adequate, while the more simple, but not quantitative Koch sedimentation method is less useful.

Zs. HORVÁTH, J. ZALA, A. SZÁNTÓ, É. KUYALEK, P. OSVÁTH,
E. K. NOVÁK, T. NAGY and Á. SZÉCSI

Examination of fungal allergens in the air of Budapest
**II. Evaluation by Burkard-trap placed onto the roof
of the Children's Sanatorium**

*B. Johan National Institute of Hygiene, Jahn Ferenc Hospital, Children's Sanatorium, Szabadsághegy,
and Plantprotection Institute, Hungarian Academy of Sciences, Budapest, Hungary*

In 1992 from March to October the most frequent potential fungal allergens (*Alternaria*, *Helminthosporium*, *Drechslera*, *Curvularia*, *Epicoccum*, *Stemphylium*,

Ulocladium, *Cladosporium*, *Pithomyces*, *Fusarium*, etc.) were detectable in the Burkard-trap planted onto the roof of the Children's Sanatorium. Fungi, being mainly plant pathogens or saprophytic species are supposed to be connected with the vegetative periods of the host plants. Generally most of potential fungal allergens seem to have a peak depending on the seasons – mainly in summer (August) with the prevalence of *Alternaria* species. The Burkard-trap is not a good detector for the numerous potential pathogen fungi (*Aspergillus*, *Penicillium*, *Trichoderma*, *Paecilomyces*, *Phoma*, *Stachybotris*, *Botrytis*, *Scopulariopsis*, *Acremonium*, *Rhizopus*, *Mucorales*, *Torula*, *Phialophora*, *Sporobolomyces*, *Aureobasidium*, etc.). The occurrence of these species, depending on the seasonality and frequency of some diseases, can be traced by only complex mycological examination (quantitative culture sampling).

A. GELETA and G. DE RUITER

Isolation of mould antigens by immunoaffinity chromatography

Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary, and Department of Food Microbiology, Agricultural University, Wageningen, The Netherlands

Mould strains belonging to *Mucorales* produce extracellular polysaccharides (EPSs) which have good antigenic properties. Some food ingredients (e.g. proteins, polysaccharides), however, also give positive ELISA with the anti-EPS thereby causing false-positive results. The aim of our work was to determine the chemical structure of the immunodominant epitopes of different EPSs which could provide good bases for the synthesis of artificial specific antigens. EPSs produced by different mucoralean strains were fractionated to two parts by an immunoaffinity column: one of them contained the immunodominant epitopes while the other consisted of the non-immunogenic fraction. Both fractions were hydrolysed to monosaccharides by methanolysis and TFA hydrolysis. The monomers were identified by HPAEC (high performance anion exchange chromatography) analysis. The mannose content of the immunodominant EPS fractions of all the 7 strains studied was extremely high; the reason for this may be that mannan domains of EPSs are responsible for the immunogenic nature of the epitopes. The epitope fraction was hydrolysed by different mannosidase enzymes which resulted the complete elimination of the immunological reaction. It is highly probable that the mannan domains of the EPSs are responsible for the epitope nature of them.

A. JAGICZA, I. VARGA, I. BÁNHEGYI, G. KUBÁNYI and G. MOLNÁR

Studies on the ultrafiltration and detoxification of tetanus toxin produced by large-scale cultivation

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Unpurified tetanus toxoid used for active immunization is a mixture of proteins with different molecular weights. In these experiments, the purification of the crude toxin by using ultrafiltration and its effect on the purified tetanus toxoid was studied. After finishing the fermentation cycle and after the total lysis of bacteria, bacteria were removed from the harvest by sterile filtration and then the highly effective tetanus toxin was immediately ultrafiltered. The toxicity of the permeate obtained at the ultrafiltration was tested. By using mouse-test, the permeate did not show any detectable toxic activity. To determine the optimal concentration of formaldehyde, the following methods were used for the detoxification of the ultrafiltered toxin. During detoxification, mouse inocuity test was used to monitor the process. After finishing detoxification, the native toxoids obtained from the ultrafiltered toxin were tested by using guinea pig inocuity tests. All methods proved to be acceptable. The results of the purification by using ammonium-sulphate suggest that the purity (Lf/mgPN) of tetanus toxoids made of ultrafiltered toxin is higher than that of the native tetanus toxoids prepared without ultrafiltration.

E. URBÁN, J. PERÉNYI, I. KÖVESDI, E. RÁCZ and J. FÖLDES

Interaction between the host cells and investigation of the free radicals and the antioxidative enzymes

Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary

In many pathological processes, in infections the production of the reactive oxygen intermediates (ROI) are considered as the reason for the damages in the organism. The production of ROI is accompanied with a repair mechanism: detoxification and raising of antioxidative enzymes. The activity of ROI and antioxidative enzymes was investigated in the peritoneal macrophage of mice, GMK and MRC-5 cell cultures infected with *Toxoplasma gondii*. In the protozoon infected cell cultures the following enzymes were measured: superoxide dismutase (SOD), catalase (CAT), glutathion reductase (GSH), glutathion peroxidase (GPO) and lipid peroxidase (LPO). The ROI (OH^- , O_2^- and NO) activity was also measured. In the infected macrophage cultures the activity of the SOD, CAT, LPO and the GSH show a peak at the 24 h. In the infected GMK cultures the SOD and CAT production reaches the peak at 48th h, and is followed by a decrease. The GSH content is growing continuously. The production of GPO starts only in the 48th h of the infections, after

72 h it decreases. The LPO activity is going on after 48 h. These experiments were completed with the measuring of the ROI (OH^- , O_2^- , and NO) activity.

AGRICULTURAL AND FOOD MICROBIOLOGY

T. V. PINTÉR

Conductance assay in food microbiology

National Institute of Food Research, Budapest, Hungary

The fully automated MALTHUS 2000 system, suits for qualitative and quantitative determination of bacteria and yeasts present in the food. Microbial growth is followed up by using differentiating nutrient media and continuous measurement of conductance. A close correlation was found between the growth curve parameters and the results of the reference method. The former were calculated by using the MALTHUS computer programme. As optical purity is not a precondition, most of the food-industrial samples can be examined. The method has been tried in examination of milk, dairy products, chocolate, cacao, fresh salad and syrups. *Enterobacteriaceae* and coliforms as well as total living count and yeasts have been included in the studies. The instrument was suitable for testing microbial growth in 120 samples at temperatures between 5 °C and 55 °C. The instrument is rather expensive, however, its functioning and the nutrient media are cost-saving; the method, being time-saving (it needs 3 to 10 h) makes a potent microbiological control possible.

K. POSTA, Cs. DOBOLYI and I. DAMJANOVA

Mycelium extraction methods (EME) for the collection of VAM extraradical mycelium

Department of Microbiology, University of Agricultural Sciences, Gödöllő, Hungary

The presence of VAM in soil usually detected by observing root colonization and identifying spores. Extraradical mycelium is rarely studied, probably because of difficulties in its extraction. Mycelium extraction was made according to Vilarino et al. (1993) as a reference method. The mycelium were collected with an external mycelium extractor (EME). The agar-film technique was studied measuring the extraradical hyphae length. The EME method seems useful in physiological studies of VA mycorrhizal fungus above all in determination its fresh or dry weight, total length and viability.

Gy. TURÓCZI and L. VAJNA

**The plant growth-promoting effect
of *Trichoderma* isolates**

Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary

It is well known that many of the antagonist microbes do not only protect the plant against different phytopathogens but promote the plant growth as well. This plant growth-promoting effect can be the result of different mechanisms. First of all, the antagonist microbe can reduce the number of ubiquitous "minor" pathogens, thus the soil and rizosphere microflora may be more favourable for the plant. The elicitor effect of either fungi or bacteria (or their cell wall components only) has been already observed as well. It means that the contact of the plant roots with these microorganisms results in resistance against pathogens even in the aerial parts of the plant. The antagonists may enhance the nutrient supplement of the plants by the transformation of mineral or organic compounds in the soil. Finally, the antagonists, like many other microorganisms are able to produce plant growth regulators, effecting the plant growth in this way. The plant growth-promoting effect of the antagonist trichodermas was observed earlier in the Plant Protection Institute in greenhouse experiments on lettuce. That is why we have decided to screen the pre-selected antagonist isolates of *Trichoderma* for their possible plant growth-promoting effect. The plants were grown in sterilized mixture of sand and vermiculite. The nutrients for the plants were supplied by mineral solution. The trichodermas were applied as soil inoculant (10^7 – 10^8 conidia/g soil) or as seed treatment combined with different carriers. Many isolates showed significant plant growth-promoting effect. They stimulated both the germination and plant growth, especially in the early stages of plant development. Some isolates, applied as soil inoculum, inhibited the plant growth. Generally, the seed treatment was more effective than the soil inoculation, many of the isolates was only effective as seed dresser.

Z. NAÁR and M. KECSKÉS

**Effect of combinations of antagonistic *Trichoderma viride*
isolates and fungicides on the growth of *Sclerotinia minor***

University of Agricultural Sciences, Gödöllő, Hungary

The possibilities of combined application of four previously selected *Trichoderma viride* strains and three fungicides (mancozeb, benomyl, and vinclozolin) against *Sclerotinia minor* were studied in laboratory experiment. The development of antagonistic fungi was inhibited at significantly different concentrations of chemicals: the LD₅₀-value of mancozeb, benomyl, and vinclozolin proved to be ~ 100.0, 0.1–1.0, and 1.0–10.0 mg per litre, respectively. The fungicide sensitivity of *Trichoderma*

strains differed only at lower doses (0.1–1.0 mg per litre). The joint effect of microbes and fungicides on the growth of phytopathogenic fungus showed significant difference at combinations. The low amount of chemicals decreased the inhibitory effect of one of four strains, whereas others were stimulated. However, combinations with higher doses of benomyl, and vinclozolin, were more effective on *Sclerotinia* than biocontrol agents. Combinations with mancozeb, benomyl, and vinclozolin hindered the growth of *S. minor* in 17.1–93.7%, 14.6–100.0%, and 88.3–100.0%, respectively. The production of *S. minor* sclerotia also decreased. No sclerotia were formed in treatment with *Trichoderma* strains alone. This effect was decreased in combinations with fungicides, resulting in the production of sclerotia: with mancozeb, benomyl, and vinclozolin 4.8–5.7, 0.0–40.2 (!), and 0.0–6.3 sclerotia per petri plates were counted.

S. NEMES, M. KONTRA and A. ZRENCSEK

**Effect of Iprodion on soil microbes, and effect of soil
microbes on Iprodion, examined under laboratory
circumstances**

*Department of Agricultural Chemical Technology, Technical University, Budapest, and Research Station
for Plant Hygiene and Soil Protection, Budapest, Hungary*

The effect of the antifungicide Rovral and that of its effective substance Iprodion was examined on several bacteria: *Bacillus subtilis*, *Bacillus cereus*, *Bacillus polymyxa*, *Pseudomonas fluorescens*, *Azotobacter croococcum* and on several fungi.

With bacteria, dilution technique with bouillon and Ashby media was used. The examined concentrations corresponded to the 1–5 kg/ha concentrations used usually on fields. The number of bacteria decreased with 2–3 magnitude even at the lowest concentration, 0.33 mg/cm³ media corresponding to 1 kg/ha in the field. Increasing the concentration the effect was less – for *P. fluorescens* we could see even some stimulation at every concentration. The effect of Rovral on fungi was examined by the means of gravimetry. Though Iprodion is usually used against *Alternaria* even at 0.33 mg/cm³ concentration, 2–3% increase was observed with our *Alternaria* sp. compared with the control (without fungicide). *Trichoderma viride* and *Mucor ramannianus* were growing even at 10 times higher concentration (2–6% of the control). In the second part of the experiments 0.33 mg/cm³ concentrations were used to see the microbial degradation. After extraction in chloroform the samples were evaporated and resolved in acetonitril:water 60:40 eluent. In HPLC with UV detector, the peak, characteristic to Iprodion (Rt:4–2) disappeared (in two weeks) and a new peak with Rt:5,3 appeared. We could see this decomposition also with TLC on Kieselgel F 254 with Bensene:methanol 95:5 eluent.

I. LENTI and S. BALÁZSY

Ovarian diseases of our shelled fruits

Department of Botanics, Teachers' Highschool, Nyíregyháza, Hungary

In the last six years we studied microscopic fungi that are able to infect the fruits of walnut (*Jugulans regia*), hazel (*Corylus avellana*) and almond (*Amygdalus communis*) in the Nyírség region (North-eastern part of the Great Hungarian Plain). The flowers and fruit of walnut are attacked by *Alternaria nucis* MOESZ and *Penicillium expansum* LINK. From the fruit of hazel 11 pathogenic fungi were isolated, *Alternaria consortiale* (THÜM-) HUGHES, *Aspergillus flavus* LINK and *Penicillium cyclopium* WESTLING having been the most common among them. Also the fruit of almond is attacked by a number of moulds: some *Penicillium*, *Aspergillus*, *Alternaria* and *Fusarium* species as well as *Trichoteceum roseum* FRIES and LINK and *Sclerotinia linhartiana* PRILL et DEL to list only the most important ones. The damage caused by fungi may be characterized by the amount of mouldy fruits, the consequent loss of weight and a considerably worsening of nutritional value.

H. E. A. F. BAYOUMI and M. KECSKÉS

Growth optimalization of some *Rhizobium* strains under ecophysiological stresses of the environment

Department of Microbiology, University of Agricultural Sciences, Gödöllő, Hungary

Limits of salts tolerance of legume as well as *Rhizobium* for successful nodulation and symbiosis are important parameters involved in salinity problem of the environment and need further more studies. In vitro experiments described here specifically address the question of the feasibility of enhancing the growth and survival of microsymbiotic N₂-fixing bacteria under salt stress of the nature by selecting salt-tolerance of *Rhizobium*. Four parameters of growth potential of *Rhizobium* strains of four cross-inoculations (*Vicia*, *Pisum*, *Phaseolus* and *Lotus*) were measured for optical density at 550 nm under the effect of five concentrations of seven macro- and five microelements in the form of chlorides in milli- and micromole, respectively, in yeast mannitol water medium at using microfermentor technique. (1) The growth of the strains was promoted in the presence of Ba⁺⁺ (100–300 mM), Mg⁺⁺ (30–40 mM), Fe⁺⁺ 200–400 µM, Mn⁺⁺ (25–200 µM) and Zn⁺⁺ (25–50 µM). (2) Cu⁺⁺ was toxic to *R. leguminosarum* strains HB-3841 and Bükköny 75/4 (bv. *viciae*) and L6 133/64 (bv. *trifolii*). (3) Ca⁺⁺, Li⁺, Na⁺ and Co⁺⁺ inhibited the growth potential of *R. loti* (Baltacim-3) and *R. leguminosarum* strains 1012 (bv. *viciae*), Bab 5/3 (bv. *phaseoli*) and L6 133/64. (4) NH₄⁺ ion was lethal to the latter strains except for Bab 5/3 that was promoted by 200 ml. (5) All of the strains required K⁺ ion except 1012 and Bab 5/3.

These results indicated that it is important to select as inoculant an effective *Rhizobium* strain for each soil type.

H. E. A. F. BAYOUMI and M. KECSKÉS

**Effect of Cu^{++} , Zn^{++} and Mn^{++} and fungicide-containing
heavy metal on the growth of some fast growing
Rhizobium strains**

Department of Microbiology, University of Agricultural Sciences, Gödöllő, Hungary

The purpose of this investigation was to develop a procedure for testing the tolerance of seven *Rhizobium* strains of four cross-inoculation groups: *R. leguminosarum* bv. *viceae* HB-3841 (Libya), Lóbab Z and Bükköny 75/4 (Hungary) and 1012 (UK) and Hungarian strains of bv. *phaseoli* (Bab 5/3), bv. *trifolii*, (Ló 133/64) and *R. loti* (Baltacim-3) to the effect of potentially hazardous four concentrations (0.1, 1, 10 and 100 mg l⁻¹) of fungicides containing heavy metals (Cu^{++} as copper oxychloride, Zn^{++} and Mn^{++} in Dithane M-22 and FL) and five concentrations of these metals in pure salts of Cl^- and SO_4^{2-} in vitro. Microfermentor technique showed the best variation in the term of strain tolerance among the examined substances. Difference in growth response of the investigated strains existed between the cross-inoculation groups. All strains were sensitive to Cu^{++} ions in pure and applied compounds. Generally, Lóbab Z and Ló 133/64 were the most tolerant and inhabitant strains to Cu compounds. Cu^{++} ions in sulphate form was significantly highly toxic to these bacteria. Dithane M-22 was more toxic to representative strains of the crossinoculation groups than Dithane FL. The significant difference at $P < 0.05$ of the interactions between the tolerance of the strains and fungicides was low and it was high between the strains and the each of fungicide concentrations. Among Zn^{++} and Mn^{++} in Cl^- and SO_4^{2-} forms, *R. leguminosarum* bv. *viceae* strains were more tolerant to these ions than the other biovars and *R. loti* strains. Lóbab Z and Bükköny 75/4 were more tolerant to Zn^{++} and Mn^{++} , respectively than the others. The growth potential of the strains declined when the concentration of the fungicides, Cu^{++} and Zn^{++} was increased in YEM broth, while it was at maximum when the Mn^{++} ion concentration was 2.747 and 5.494 mg l⁻¹ in Cl^- and SO_4^{2-} forms, respectively. The strains preferred to grow in a medium containing MnSO_4 than MnCl_2 .

J. KUKOLYA and Cs. DOBOLYI

**Effect of a cellobiase inhibition on the cellulase activity of
Cellulomonas sp.**

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The investigated *Cellulomonas* sp. produced 45 kD, surface bound, true cellobiase (EC 3.2.1.21.). Kinetic studies showed that cellobiase activity was inhibited competitively by δ -glucono-lactone, with a K_i value of 10^{-5} M. So glucono-lactone proved to be a powerful inhibitor for the inactivation of the cellobiase enzyme. In micro-fermentation experiment we used six types of media parallel to demonstrate the effect of the inactivated cellobiase on the cellulase activity. The 1% methyl-cellulose-containing medium was supplemented with the following components: cellobiose, cellobiose+lactone, glucose, glucose+lactone and lactone. The inoculum of *Cellulomonas* was grown in 1% glycerol-medium for 24 h, then we inoculated. The methyl-cellulose broth with membrane filtered and washed bacterial mass (10^8 cells/ml). The actual reducing-sugar level was measured with DSA method, pH, bacterial number and OD; β -glucosidase and total cellulase activity was also continuously followed during the incubation period (12 h). Glucono-lactone affected negatively the bacterial number, β -glucosidase and cellulase activity in all measured samples. Cellulase activity was 3–4 fold lower in the lactone-containing broth. The greatest cellulase activity was determined after a 5 h incubation in the cellobiose-containing media. From the results of our experiments we concluded that the role of cellobiase enzyme is not only to prevent the accumulation of cellobiose which acts negatively both on the exo- and endoglucanase, but besides it may act on cellulase induction, too.

I. VÖRÖS, K. KÖVES-PÉCHY and J. SZEGI

**Dual symbiosis of VAM fungi and *Rhizobium*
in recultivated mine spoils of Visonta**

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Budapest, Hungary*

The possibilities of recultivation in open cut lignite mine spoils (andezite tuff, yellow sand, yellow and grey clay) were studied. Incidence of VAM fungi and rhizobium nodules on the roots of *Pisum sativum* was studied after 8 and 15 years of cultivation in Visonta (Hungary). Effect of rhizobium inoculation and interaction of both symbionts on the plant production were also examined in the following variations (control, NPK fertilizer, NPK+lignite, NPK+straw, sewage sludge). VAM fungal infection frequency (F%) on the pea roots showed high value in most of the cases with the spoils containing organic materials. Between plant production and F% value a

positive relationship could be often observed. The number of spontaneous rhizobium nodules was relatively low, so green yields of pea was not influenced by them. Both were, however, enhanced after inoculation with *Rhizobium leguminosarum* bv. *viceae* strains. Although the VAM infection frequency has also been increased after rhizobium inoculation, it did not cause any stimulating effect on the green yields of pea. Organic materials had a favourable effect on the recultivation processes and increased the fertility.

G. VERECZKEY and M. GOSZTONYI

Erythadene content of *Lentinus edodes*

Department of Bioengineering, Central Research Institute of Food Industry, Budapest, Hungary

The fungus *Lentinus edodes* (Shiitake) has been known in China and Japan for 2000 years. After World War II, various technologies were developed for its cultivation, and its wide-scale preparation was dealt with in the USA, Canada and Europe. Shiitake may be of interest as delicacy and also as a fungus to be used for therapeutical purposes. Numerous bioactive substances (e.g. erythadene i.e. 2,3-dihydroxy-4-(9-adenyl butyric acid) have been isolated from its pistle. It has been shown that both total blood cholesterol and LDL are suppressed by erythadene treatment.

To find this bioactive substance in the mycelium of the fungus, sufficient amount of mycelium was prepared in submerged culture. Cultures containing malt juice (3 or 5%) and yeast extract (0.2%) in 100 ml medium of pH 4.7 were shaken at 100 rpm. Pellets of uniform dispersion developed. After subsequent fermentation for two weeks, 0.4 to 0.8% and 0.3 to 0.5% biomass was yielded from the Czech and the German strain, respectively. In the Biostat apparatus, the dry biomass grew up to 0.6% during one-week fermentation in a medium containing 0.13% of diammonium tartarate. To examine erythadene, we prepared 1 g of the pure substance by organic synthesis. The dried mycelium was extracted with tenfold amount of 80% ethanol, then the extract was purified in several steps by ion-exchange chromatography (Amberlite R-120 H⁻; Amberlite IRA-400 OH⁻). To identify the substance, the GC-MS and LC-MS techniques were used.

B. BÍRÓ, T. SZILI-KOVÁCS and J. SZEGI

**Antagonism among microorganisms isolated from the
rhizosphere of wheat and maize**

*Research Institute for Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences,
Budapest, Hungary*

Antagonist effect of some fungal and actinomycete strains was investigated on N_2 -fixing and scavenger bacteria originating from the hystosphere and rhizosphere of wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.). Over the native rhizosphere bacteria the competitiveness of other authentic *Azospirillum*, *Flavobacter* and *Arthrobacter* diazotroph strains was also checked by agar disc method. Almost 70% of the microscopic fungi and actinomycetes have a significant antagonistic effect against the diazotrophs. At the same time, 90% of the scavenger bacteria (that are not N_2 -fixers) suppressed the growth of *Trichoderma*, *Fusarium*, *Penicillium* and *Streptomyces* colonies. Regarding the extent of antagonism, the strains of actinomycetes behaved more homogeneously than the strains of microfungi mentioned above.

S. TÍMÁRI, E. NÓTÁS, K. DEBRECZENI, Z. NAÁR, M. KECSKÉS, Á. BÁLINT
and GY. HELTAI

**Investigation of nitrification activity in lessivaged brown
forest soil (LUVISOL)**

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Sciences, Gödöllő, and Agrochemistry Institute, Pannon University of Agricultural Sciences, Keszthely,
Hungary*

Under greenhouse conditions the dynamics of nitrification activity was studied between 0–50 days on the basis of four sampling time in lessivaged brown forest soil (pH (H_2O)=7.1) and humus content = 1.91%). The test plant was *Zea mays*. The influence of moisture of the soil (water holding capacity = 60–70% or 70–90%) and potassium-nitrate and ammonium-chloride fertilizers (application rate: 500 kg N per ha) was investigated. Samples were taken four times on days 5, 10, 20 and 45 of the maize's emergence. Measurements were performed from air-dried soil samples. Stephenson's nutrient solution was applied to the determination of the nitrification activity. This solution contained ammonium-sulphate. Under sterile conditions 1 g fresh (moist) soil sample was weighed into the test tube and 10 ml Stephenson's solution was added. This suspension was shaken mechanically for 21 days on 25 °C. The NH_4 -N and NO_3 -N content of the suspension was determined by Bremner's method. From the decrease of the amount of ammonium-nitrogen was concluded the increase of nitrification activity. (1) The nitrification activity changed significantly as a function of time in the control and nitrogen treated samples equally, while there were no significant differences at different moisture levels. (2) The maximum activity was

measured between 10–20 days and the minimum activity on days 5 and 45. (3) The activity values were the smallest of potassium-nitrate treatment. (4) Significant differences were not obtained between the control and ammonium-chloride treated soil samples taken simultaneously. (5) The amount of $\text{NO}_3\text{-N}$ changed significantly as the function of time. The maximum values were measured on day 20. (6) Medium negative correlation ($r = -0.546$) was shown between $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ content of the soil suspension.

K. POSTA and Gy. FÜLEKY

**Effect of phosphorus fertilization and root density
on mycorrhizal colonization of maize**

*Department of Microbiology, and Department of Soil Sciences and Agrochemistry, University of
Agricultural Sciences, Gödöllő, Hungary*

The influence of root density and phosphorus fertilization on mycorrhizal colonization of maize plants was studied in sandy soil (Órbottyán, Hungary) using phosphorus fertilization (0, 50, 250 mg P/kg soil). The soil was left either uninoculated or was inoculated prior to planting with rhizosphere microorganisms together with VA mycorrhizal fungus. Effect of root density was investigated by using different pots of the following sizes: 0.5, 2.0, 5.0 and 10.0 litres. VAM inoculation usually increased maize dry matter production in all pot sized used, but the magnitude of the response to VAM was greater in the largest pots. Increasing the pot size resulted in decreased root density in every case. At low phosphorus fertilization the root:shoot ratio was small (mean value: 0.26) in mycorrhizal plants and higher (mean value: 0.35) in non-mycorrhizal plants. It was equalized at higher phosphorus fertilization. Tissues of VAM plants accumulated almost three-times more phosphorus than non-VAM plants and this decreased with the increasing of soil phosphorus.

MYCOLOGY AND INDUSTRIAL MICROBIOLOGY

A. JEKKEL, A. ANDOR, É. ILKŐY, Gy. HORVÁTH and G. AMBRUS

**Microbial degradation of plant sterol side chains
by a recombinant *Mycobacterium* sp. strain**

Institute for Drug Research Ltd., Budapest, Hungary

About 10 years ago the time was ripe for the development of genetic manipulation on mycobacteria, with many applied objectives. The first is medical, stimulated by the fact that mycobacteria are responsible for two of the world's most

important bacterial human diseases, tuberculosis and leprosy, as well as for widespread animal infections. The second is utility of various non-pathogenic mycobacteria in the field of industrial bioconversion of steroids. From the eighties the industrial synthesis of the majority of steroid drugs is based on the microbial transformation products obtained by the removal of the side chain of the cheap and readily available sitosterol. In recent years our group has isolated mutant and recombinant strains which can bioconvert the different plant sterols (ergosterol, stigmasterol, sitosterol, campesterol) into new transformation products having partially degraded side chain. The structures of five new 26-oxygenated sterol transformation products and two novel degradation products having 23,24-dinorcholane side chain were elucidated by UV, IR, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and mass spectroscopy.

T. PUSZTAHELYI, I. PÓCSI and A. SZENTIRMAI

**Regulation of N-acetyl- β -D-hexosaminidase formation
in the fungus *Penicillium chrysogenum***

Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, Hungary

Physiological studies on the fungus *Penicillium chrysogenum* have shown that the N-acetyl- β -D-hexosaminidase formation of the fungus is regulated by glucose catabolite repression. The enzyme formation is influenced, besides the glucose content of the system, by the age of the culture. The intracellular enzyme activity reaches its peak by the end of the stationary phase of growth, then, during progressive lysis, the enzyme is passively released into the outer space, where it is inactivated, probably on the effect of proteases released simultaneously. The regulation of enzyme formation might be similar to the formation of chitinases, in the course of which also glucose catabolite repression has been detected. The intracellular activity of the enzyme in the exponential phase of growth of the cell mass is negligible, therefore, its primary role in growth seems improbable. The extracellular enzyme activity reaches its peak in the dying culture. Thus, the enzyme's role in the external feeding of the fungus is also improbable. The results obtained up to now agree well with the assumption that the fungal N-acetyl- β -D-hexosaminidase may contribute to the mobilization of the reserve feeding substances of the fasting microorganism, possibly by decomposition of the chitinoligomers formed in the course of cell wall degradation.

F. KEVEI, J. VARGA, Cs. VÁGVÖLGYI, J. KUCSERA, Á. NAGY, I. PFEIFFER
and L. FERENCZY

**Interpretation of compatibility relations among black
Aspergilli on the basis of their molecular characterization**

Department of Microbiology, A. József University, Szeged, Hungary

High degree of incompatibility could be detected in parasexual crosses attempted not only in interspecific combinations, but in pairing of single isolates of certain species. Large numbers of collection strains and wild isolates were studied to find common molecular characters. On the basis of investigations of polymorphism of nuclear ribosomal RNA genes, karyotypes, RFLPs of their mitochondrial genomes, isoenzyme variations and the presence of dsRNA viruses, several groups can be distinguished in each species. Relations between different groups can be characterized by full incompatibility in respect of nuclear complementation. Among certain heterokaryon incompatible strains belonging to the same molecular marker groups, the protoplast fusion may result in some non-conventional parasexual hybrids. Applying drug-resistant mitochondrial mutants mitochondrial recombinations can be observed under strong selection pressure, without double nuclear control. Simultaneously, virus transfer can also be detected in incompatible connections under controlled experimental conditions.

I. BALOGH and A. MARAZ

Detection of STA genes by labelled DNA probes in yeasts

Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary

Yeast strains belonging to the *Saccharomyces cerevisiae* race *diastaticus* are able to produce extracellular glucoamylase enzymes. The polymorphic *STA* gene family (*STA1*, *STA2* and *STA3*) is responsible for coding the enzymes. These genes are located on different chromosomes: *STA1* is on chromosome IV., *STA2* is on chromosome II., whereas *STA3* is on chromosome XIV. The chromosomes of yeasts can be separated by pulsed-field gel electrophoresis (PFGE). In our work a more developed version of this technique the rotating-field gel electrophoresis (RFGE) was used. The 17 chromosomes of *S. cerevisiae* gave 15 DNA bands while the chromosomes of the *diastaticus* strains were separated into 15 or 16 distinct bands. The chromosomal patterns of the different *diastaticus* strains differed from each other and also from that of the non-amylolytic *S. cerevisiae* strains (CLP = chromosome length polymorphism). The karyotypes of the different amylolytic yeast strains produced by sexual and somatic hybridization were determined. A 1.45 kb long sequence of the *STA2* gene was used as a probe for the detection of the *STA* genes. The probe was labelled with a non-radioactive (DIG) DNA labelling and detection kit (Boehringer-

Mannheim). The chromosomes separated on the gel were transferred into a positively charged nylon membrane by Southern transfer and after that they were hybridized with the DIG-labelled *STA* gene probe. The *STA* genes were localized on the chromosomes with the help of different marker genes.

A. POMÁZI, A. WITTNER and L. HORNOK

**Classification of physiological races in filamentous fungi
by random amplification of polymorphic DNA**

Agricultural Biotechnology Centre, Gödöllő, Hungary

Two types of within-species subgroups are known in filamentous fungi, formae speciales, pathogenic to particular host plant species, and races, that form a more narrow unit infecting only certain cultivars. The various formae and races are morphologically uniform, their biochemical characteristics are also very similar, the only significant difference among them is their specific pathogenicity. These subgroups are identified in pathogenicity tests and on the basis of vegetative compatibility. However, pathogenicity tests are time consuming and greatly influenced by environmental conditions, while vegetative compatibility grouping is hindered by self-incompatibility and inbreeding efforts of certain strains. Thirty-eight strains of *Fusarium oxysporum* f. sp. *pisi*, the causal organism of pea wilt were investigated, partly local isolates, partly international "authentic" strains with tentative race affiliation. As the proper identification of these isolates were impossible either in pathogenicity experiments or by vegetative compatibility grouping, random amplification of polymorphic DNA, a PCR-based technique was used for their classification. A total of ten synthetic oligonucleotide primers were tried and five of them proved to be suitable for differentiation. Remarkable specificity was revealed by using the OPE-11 (GAGTCTCAGG) primer. The present subdivision of this pathogen into six races was unjustified by these experiments. The whole population of *F. oxysporum* f. sp. *pisi* actually comprises two main groups, one of them is formed by the majority of the local isolates and some foreign ones from North-America and Holland reported to belong to races 1, 2, 4 and 6, while the second contains three Hungarian strains together with race 1 and 2 strains from the US and Britain.

B. NAGY, É. TÁBORHEGYI, A. WITTNER and L. HORNOK

Variation in electrophoretic karyotypes and mycotoxin profiles among *Fusarium sporotrichioides* strains

Agricultural Biotechnology Centre, Gödöllő, Hungary

Fusarium sporotrichioides is a secondary parasite of cereals and stored grains producing a number of dangerous mycotoxins, trichothecenes and zearalenones. There are great variations in toxin profiles within species, the individual strains produces different types and quantities of mycotoxins. Changes in toxin producing ability of isolates maintained in cultures for prolonged periods are frequently observed; genetical and molecular backgrounds of these changes are largely unknown. Electrophoretic karyotypes for 25 isolates of the species were identified; the haploid genome of the fungus is about 30 Mb, the majority of the chromosome-sized DNA is concentrated in two large blotches around the 6.5–7.0 Mb and 5.4–5.6 Mb range. The number of normal-sized chromosomes is estimated to be 4–5. *Tox5*, a gene coding for trichodiene-synthase, the basic enzyme for trichothecene biosynthesis was successfully localized on the 5.4–5.6 Mb DNA band in all strains. Karyotype variability among strains was observed in the region of the small-sized "B"-chromosomes. All isolates contained mini-chromosomes ranging between 0.4 and 1.7 Mb, in numbers varying from one to three. Six different A-type trichothecenes (T-2 toxin, HT-2, neosolaniol, iso-T-2, acetyl-T-2 and T-2-tetraol), as well as zearalenone were identified by gas-chromatography in these strains in various patterns and quantities. Relationships between karyotype differences, chromosome rearrangement and variable toxin profiles are discussed.

Cs. FEKETE, I. PAPP, G. GICZEY and L. HORNOK

Molecular characterization of double-stranded RNA containing *Fusarium poae* strains

Agricultural Biotechnology Centre, Gödöllő, Hungary

When *Fusarium poae*, a fungus pathogenic to the inflorescence of small-grain cereals were surveyed the majority of nearly 50 strains was found to contain double-stranded RNA (dsRNA) elements. The number of these genetical elements varied from one to ten depending on strains and their sizes were also different. The dsRNA patterns were strain specific and remained stable after repeated subculturing of the fungi. Strains of the pathogen that contained dsRNA elements were similar to the dsRNA-free isolates and showed no morphological or physiological aberrations. Strains with different dsRNA patterns were subjected to molecular characterization. RAPD (random amplification of polymorphic DNA) profiles as well as electrophoretic karyotypes were compared and restriction fragment length polymorphisms (RFLPs) were detected by

using a cloned gene (*Tox5*, coding the basic step of trichothecene biosynthesis) or different random probes. Strong correlation was found between these molecular markers and the dsRNA patterns of the strains explaining the high stability of the latter. Of the eight random probes isolated by this group seven proved to be small copy number sequences, while one was found to be a high copy number repeat units. This highly specific probe was localized on a small-sized B chromosome.

T. HEVÉR and F. KEVEI

**Investigations on compatibility relations among
Aspergillus niger strains**

Csongrád County Institute, National Public Health Service, Szeged, and Department of Microbiology,
A. József University, Szeged, Hungary

The individual isolates of the species of black *Aspergillus* (sectio Nigri) exhibit a wide range of genetic diversity. *A. niger* is named as species aggregate in which the strains examined consist of several groups on the bases of the characterization of their molecular markers. In respect of parasexual hybridisation a high degree of incompatibility exists among the isolates belonging to the same molecular group. Applying protoplast fusion technique new hybrid type colonies could be isolated from those combinations where the regular parasexual cycle following hyphal anastomosis is excluded. Fusion colonies appeared under strong selection pressure using auxotrophic mutants. The new hybrid phenotypes isolated after subsequent steps proved to be stable on non-selective conditions. The isolation processes and the possible genetic constitutions and behaviour of the hybrids will be discussed.

J. KUCSERA, J. CSIHA, A. NAGY, I. PFEIFFER and L. FERENCZY

Interspecific protoplast fusion of *Saccharomyces cerevisiae* and *Saccharomyces kluyveri*

Department of Microbiology, A. József University, Szeged, Hungary

Saccharomyces cerevisiae and *Saccharomyces kluyveri* are the most distantly related members of the genus. Although their mating factors act on both yeasts, they do not mate with each other. In spite of these, hybrid formation could be achieved via protoplast fusion. PEG-induced protoplast fusion of auxotrophic mutants resulted in hybrids with a frequency of 1.2×10^7 /plated protoplasts. Their cytological and biochemical analyses revealed that the resulting hybrids were phenotypically similar to *S. kluyveri*. Electrophoretic karyotyping showed that in some of the fusion products had a total chromosomal set from *S. kluyveri* and one or even two chromosomes from *S. cerevisiae*, while in others chromosomal DNA characteristic of *S. kluyveri* could

only be detected. The inheritance of the killer phenotype of *S. cerevisiae* was also studied in the hybrids. None of them had killer activity. dsRNA plasmid isolation demonstrated that they contained only LdsRNA. MdsRNA, which is responsible for the toxin production, could not be detected.

Zs. HAMARI, F. KEVEI and J. VARGA

Characterization of *Aspergillus japonicus* and *Aspergillus carbonarius* strains based on mitochondrial DNA RFLPS

Department of Microbiology, A. József University, Szeged, Hungary

Mitochondrial DNAs (mtDNAs) of wild-type isolates and collection strains belonging to the species, *Aspergillus japonicus* and *Aspergillus carbonarius*, have been analysed by digesting with *EcoRI*, *HaeIII*, *BglII* and *PvuII* restriction enzymes. Both species were found to have larger mtDNAs than that found in *Aspergillus niger* (30–32 kb). The size ranges were 42–50 kb and 40–43 kb in the cases of *A. japonicus* and *A. carbonarius* strains, respectively. The thirty-six *A. japonicus* strains examined belong to seven different RFLP groups, while all except one of the eleven examined *A. carbonarius* strains showed the same pattern with all enzymes applied. Hybridization experiments were carried out with cloned *Aspergillus nidulans* mitochondrial DNA fragments (*cox1*, *atp9*, *m1* genes). The results obtained suggest that some of the RFLP groups observed in the case of *A. japonicus* are very closely related, while some others showed completely different hybridization patterns. The nuclear ribosomal repeat units of the *A. japonicus* and *A. carbonarius* strains were compared by hybridizing the *A. nidulans* ribosomal RNA complex to *SmaI* digested nuclear DNA patterns of the strains. The patterns obtained were specific to each species.

J. TORNAI

Significance of active fructose transport in the *Saccharomyces sensu stricto* group

Department of Microbiology, University of Horticulture and Food Technology, Budapest, Hungary

In the past decades, separation or lumping-together species of the *Saccharomyces sensu stricto* group have been a hot issue. Using conventional phenotypic identification, delimitation of species within the group cannot be done satisfactorily. On the basis of nuclear DNA–DNA reassociation studies, following distinct species were singled out: *S. cerevisiae*, *S. paradoxus*, *S. bayanus* and *S. pastorianus* (Vaughan–Martini and Martini, 1987). In 1990, Rodrigues de Sousa et al. tested fructose proton symport activity in *Saccharomyces* species. They reported significant differences, and concluded that active fructose transport is an excellent

phenotypic character that correlates with species delimitation based on the genotype. Here, we present preliminary results on fructose proton symport measurement in 72 strains belonging to the *Saccharomyces sensu stricto* group. All strains were obtained from the National Collection of Agricultural and Industrial Microorganisms (NCAIM). They were identified earlier by using established conventional methods. Our results show that, based on their fructose proton symport activity, 12 strains do not correlate with their original classification. We assume incorrect identification. Current genetic studies will help decide the significance and usefulness of active fructose transport in separating members of the *Saccharomyces sensu stricto* group.

Á. NAGY, Cs. VÁGVÖLGYI and L. FERENCZY

Electrophoretic karyotyping of *Mucor* species

Department of Microbiology, A. József University, Szeged, Hungary

Pulsed field gel electrophoresis (PFGE) techniques have advanced rapidly in recent years resulting in the resolution of chromosomal size DNA molecules up to 12 megabase pairs (Mb) in size. As the most versatile pulsed-field system, the Contour-Clamped Homogeneous Electric Field (CHEF) gel electrophoresis has been used to produce highly uniform electric field in order to establish good DNA resolution and to determine karyotypes of strains belonging to different *Mucor* species. In spite of their increasing biological and economical importance, knowledge of their genetic systems (size and organization of the nuclear and mitochondrial genomes) is rather rudimentary. Intact chromosomal DNA was prepared from mycelial protoplasts and subjected to CHEF electrophoresis under various conditions. Optimized parameters of running allowed the separation of different chromosomal DNA molecules. Five to eight chromosomal bands have been obtained, some of them probably containing more than one different chromosomal DNA molecules of the same size. Using video densitometric analysis and the chromosomal DNA of *Schizosaccharomyces pombe* as size standards, the sizes of DNA in *Mucor circinelloides* f. *lusitanicus* (ATCC 1216b) chromosomes found to be between 1.7 and 8.1 Mb with a total genome size of approximately 39 Mb.

K. HALMY and I. HORVÁTH

**Occurrence of *Candida* in different forms
of human psoriasis**

*Gy. Kenézy Hospital, Debrecen, and Hajdú-Bihar County Institute, National Public Health Service,
Debrecen, Hungary*

Candida counts were studied by quantitative and semi-quantitative methods in the mouth cavity and in the intestinal flora of 46 patients suffering from extensive psoriasis (including 20 seborrhoeic, 8 erythrodermic cases and 18 patients with large plaques) as well as of 20 persons in good health for control. The investigations were extended to determination of antibodies to *Candida albicans* by ELISA, counter immunoelectrophoresis and immunodiffusion methods.

In extensive forms of psoriasis, candida counts increased significantly in relation to the control in the intestines, but not in the mouth flora. Differences among types of psoriasis concerning the occurrence of candida were not found to be significant. The presence of antibodies to candida and positive cultivation findings showed a close correlation. Positive results were produced mostly by Ouchterlony's immunodiffusion method, and this same method proved to be the most specific, too. Elimination of the pathogens in patients, whose intestinal tract was found repeatedly infected by a great number of candida, was tried by prescribing nystatin treatment. Flare-up of psoriatic symptoms may have been reduced by this treatment with good results.

J. SERFÓZÓ and K. HALMY

**Reaction of *Candida albicans* blastospores
on the treatment of fluconazole and itraconazole**

*Department of Comparative Animal Physiology, L. Kossuth University, Debrecen, and Hajdú-Bihar
County Institute, National Public Health Service, Debrecen, Hungary*

The blastospore is one of the developmental forms of *Candida albicans* having large vitality. Their growing and development is influenced by antimycotics through the cell membrane genesis because these drugs can inhibit the synthesis of membrane steroids. The yeast cells are able to protect against the necrotic effects. It seems that their protective quality is connected with a tubulo-vesicular system which is hypertrophied by different doses of antimycotics. The different doses (10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} M.ml⁻¹) of fluconazole and itraconazole induce the hypertrophy in this system, too. The occurrence is manifested in the increasing the number and size of vesicular elements of tubulo-vesicular system. The detoxication can operate very excellently in the blastospore because neither cell nor tubulo-vesicular system's membrane are necrotized during the effect of antimycotics. Consequently, it was not accidental that cellular necrosis could not be investigated.

A. KÁLMÁN and B. LENKEY

**Effect of heavy metal ions on the growth
of *Saccharomyces* sp. and on the function
of its 3-hydroxysteroid oxidoreductase enzymes**

Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, Hungary

It is well known that organisms require various heavy metal ions in a certain quantity, but higher concentrations may change morphology and reproduction of cells and the intensity and nature of their metabolism. In our experiment we used a *Saccharomyces* sp. capable of hydroxylating steroids in 14- α or 17- β positions. We examined the growth of our organism in Sabouraud liquid medium (4% glucose, 1% pepton) and by the method of immobilization in Sabouraud agar in the presence of Fe^{++} , Mn^{++} , Co^{++} , Cu^{++} and Cr^{+++} singularly between 3×10^{-3} – 5×10^{-2} mol/dm³ concentration. On Sabouraud agar plates this yeast grows at the concentration of 4×10^{-4} mol/dm³ Co^{++} , 1.2×10^{-2} mol/dm³ Cr^{+++} and 2.5×10^{-2} mol/dm³ Mn^{++} . We found that the growth of yeast was limited by Co^{++} , Cu^{++} and Cr^{+++} in Sabouraud liquid medium Fe^{++} and Mn^{++} caused only inhibition at higher concentrations. Growth of immobilized yeast is similar. Hydroxysteroid-oxidoreductase enzymes reduced methyl-secodione (3-methoxy-8,14-seco-1,3,5(10),9(11)-estrateraen-14,17-dione) as substrate when less than 5% 14-hydroxi and 30–40% 17-hydroxi derivatives formed. In the presence of heavy metal ions forming of reduced product decreases. Under immobilized conditions, the growth of yeast is still considerable at 6×10^{-13} mol/dm³ Cr^{+++} concentration and it has reduction capacity, too. We could increase the Cr-tolerance of our yeast to 2.5×10^{-2} mol/dm³ concentration but this Cr-tolerant yeast lost the ability of forming 17-hydroxi derivative of substrate.

E. H. ASHOUR and E. K. NOVÁK

**Cobalt binding of *Saccharomyces cerevisiae*
and *Rhodotorula glutinis***

B. Johan National Institute of Hygiene Budapest, Hungary

For the bioaccumulation of heavy metal ions polluting the environment fungi seems to be profitable candidates. Therefore the Co^{++} tolerance and binding of *Saccharomyces cerevisiae* and *Rhodotorula glutinis* were compared. In preliminary studies resting cells of *S. cerevisiae* was the best absorber (0.5 g w/w cells bound 99% of 10 ml 0.05 mM Co^{++} within 3 h, determined by AAS), while with growing cells *R. glutinis* was the most tolerant. Details of Co^{++} uptake by resting cells of the two yeasts in saline were studied with radioactive labelling. Cobalt uptake kinetic of both species was measured with an initial concentration of, besides 1.0 and 0.3, mainly 0.1 mM CoCl_2 , and 0.04 g w.w./ml biomass content. The data indicate that the uptake of this

heavy metal ion is quick (the major part of absorption with 0.1 mM proceeds in the first 30 min), while the equilibrium with higher concentrations is achieved within 2 h. *S. cerevisiae* resting cells bound ca. 80%, while *R. glutinis* reached 40% at 30 min. Addition of glucose and/or peptone stimulated the uptake. Low pH, the respiration inhibitor azide, or the detergent cetylpyridiniumbromide markedly inhibited the process, but partial inhibition was detected by the uncoupler 2,4-DNP. Divalent cations Zn^{++} or Ni^{++} inhibited competitively. Co^{++} uptake of bath strains is suggested to be an "pH sensitive" active (uphill) transport.

E. H. ASHOUR, T. NAGY, B. SCHOKET and E. K. NOVÁK

**Uptake of Cr^{VI} by *Saccharomyces cerevisiae*
and *Rhodotorula glutinis***

B. Johan National Institute of Hygiene, Budapest, Hungary

Continuing our experiments in the subject of heavy metal ion binding on fungi, being very important in the environmental protection, we have studied the chromate binding of the resting cells of the yeasts mentioned above with ^{51}Cr marker in various circumstances. The growth of our strains in Sabouraud glucose broth, however, was only partially inhibited by the presence of 0.1 mM CrO_4^{2-} . The ion uptake of the cells is directly proportional to the outside concentration within the range 0.1–10 mM, but above it declines, because of a supposed saturation in the uptake kinetics. The pH-s below 4 promoted, while above 4 decreased the uptake. Addition of glucose increased, while that of peptone decreased the process. The presence of SO_4^{2-} is slightly competitive in the case of *S. cerevisiae*, and is moderately in the case of *R. glutinis*. Among the antimetabolites (1 mM) NH_2OH is stimulatory with *R. glutinis*, only 2,4-DNP, and cycloheximide are definitely inhibitory with it, while ICH_2CONH_2 , N_3^- , and the detergent cetylpyridinium-bromide are less inhibitory, however, they are stimulatory in case of *S. cerevisiae*. Comparison of the effects (positive or negative) on the Cr^{+++} and CrO_4^{2-} uptake of both 4 strains showed that the same tendency is true only for 2,4-DNP and NH_2OH , but not for all the others.

J. HEGÓCZKI

Production of yeasts enriched with micro-elements

Department of Agricultural and Chemical Technology, Technical University, Budapest, Hungary

Yeasts enriched with micro-elements (MEs) were prepared. Yeasts under well-defined conditions are capable of being enriched with certain MEs up to several times of their normal levels and of fixing the MEs with organic bonds. MEs from organic sources are much more suitable and advantageous for the animal (including human)

organism those obtained from inorganic sources. The biotransformed MEs in the yeasts have better absorption quotients, they are less toxic, taste better and are easier to dispense. The full-value proteins and high vitamin content of the yeast contribute to the advantageous effects of the biotransformed MEs. Enrichment of yeasts leads to ME sources of "natural" origin.

The ME concentrations in ppm and the degrees of enrichment in seven preparations (normal yeast/enriched yeast) were as follows: Fe 60/1000–10 000; Cu 27/500–1000; Zn 150/500–5000; Se 0.4/1000–6000; Zr 0.01/800–5000; Al 20/500–1500; Bi 0.01/200–1200.

The yeasts enriched with MEs and their water-soluble extracts can be applied as additive in almost all areas of food industry. The first product being under preparation in Hungary will be a curative bread containing yeast enriched with selenium. Also sweet industry would utilize similar products. Paramedical products may be mentioned as a further area of appliance of enriched yeasts. The EPASEL capsule containing polyunsaturated fatty acids (EPA and DHA), selenium-enriched yeast and vitamin E has been put into circulation by the BIOREX Co in Hungary and elsewhere in Europe. A similar preparation (KONDI) is produced by the EPAKER Ltd. The latter contains zinc-enriched yeast and lecithin of plant or animal origin.

A. ANDOR, T. KIESER and A. JEKKEL

**Construction of cosmid gene libraries from mycobacteria
that express important sterol-modifying enzymes**

Institute for Drug Research Ltd., Budapest, Hungary, and John Innes Institute, Norwich, UK

Mycobacteria are non-sporulating Gram-positive bacteria with DNA of high G+C content. Many mycobacterial species can utilize the readily available plant sterols as a sole carbon source. In the course of our research several mutant and recombinant *Mycobacterium* strains have been isolated that, unlike most other members of the species, degrade the sitosterol onyl partially and by doing so create useful intermediates for the chemical synthesis of steroid drugs. Some of these strains contain enzymes that make the particularly useful hydroxylation at the C₉ position of the steroid skeleton and degrade the side chain of the sitosterol partially or completely or create a double bond between C₁ and C₂ in addition to the total degradation of the side chain. It was our aim to clone the genes for these activities and also genes involved in sitosterol side chain degradation. For the cloning of these genes *Mycobacterium* host strains are available that lack these activities. Genomic gene libraries were constructed from 9 α -hydroxy-4-androstene-3,17-dione producing *M. roseum* and 1,4-androstadiene-3,17-dione producing *M. phlei* in two cosmid vectors; pYUB18 replicating autonomously in mycobacteria and pYUB268 integrating into the chromosome site specifically.

S. SZOBOSZLAY, G. TÖRÖK, B. KRISZT, S. RUZS-MOLNÁR, M. SAJGÓ, L. LAKICS, L. BÉLAFI and J. HERENDI

Stimulated biodegradation of hydrocarbons

*Department of Chemistry, Department of Biochemistry, University of Agricultural Sciences, Gödöllő,
Department of Oil-biology, MOL Co., Komárom and Application- and Systemtechnical Research
Department, Hungarian Oil and Gas Research Institute, Veszprém, Hungary*

The objective of our investigation was to degrade hydrocarbon pollutions by biotechnological processes: (i) in soils in situ (to 50 cm depth); (ii) by compost-making technologies; (iii) in réservoirs, by washing and fermentation technologies. For experimental purposes mixed bacterium cultures – isolated from CH polluted areas – were used, including non-pathogenic *Pseudomonas*, *Micrococcus*, *Nocardia* and *Rhodococcus* species as predominant ones. After agrotechnical preparation, the oil polluted material was inoculated with 5–10 L/m³ inoculum containing 109 microbes/cm³. According to our investigations, 80–90% of the oil in the inoculated material was degraded within 3–180 days, depending on the character of treatment. It was shown by gas chromatographic and infrared spectrophotometric analyses that a significant proportion of n-paraffins and PAH-s was eliminated from the treated material by the microbes deposited at the National Collection of Agricultural and Industrial Microorganisms under numbers NCAIM 001190, 001191, 001192.

T. NAGY, E. K. NOVÁK, J. ZALA and Zs. HORVÁTH

Prototype of and EXPERT SYSTEM in the mycology

B. Johan National Institute of Hygiene, Budapest, Hungary

EXPERT SYSTEM program for the identification of the pathogenic fungi has been developed for mycological routine work. EXPERT(s) can define a special graph with selection lists, texts, pictures for the users. There can be selected max. 8 selection items in a selection list. After selecting an item, the user can reach a new selection list with text and picture information. The user can select, among the items and “travel” to a certain graph point defined by the EXPERT with selection list, text, and picture help. The user has several possibilities (word, text, picture) to view a special graph point with complex information, when it is necessary. It's possible to continue the searching from this point with selecting a new item. It's also possible to search backward from any given graph point. The point that can be searched backward is where you can reach the actual graph point (selection list) where you were. Several “backward search” points can be reached, if any. The logical path can replay by the history (in case of “backward search” an interrupt ensures that only the constructive logical path stored by the history). There is also an on line help for the user(s).

É. TAS, A. SAANO, S. KAIJALAINEN and K. LINDSTRÖM

Isolation of a strain-specific DNA probe by subtraction hybridization

Department of Applied Chemistry and Microbiology, Division of Microbiology, University of Helsinki, Helsinki, Finland

DNA hybridization is considered to be a useful tool for identification and detection of bacteria from different backgrounds (e.g. from soil or nodules). For this purpose specific probes are needed. Subtraction hybridization is a powerful method for isolating DNA fragments that differ in abundance between two DNA mixtures. The total genomic DNA of two different bacterial strains are fragmented and one of them is labelled with biotin. After subtraction hybridization the biotinylated fraction together with the hybridized homolog non-labelled DNA is separated. The remaining DNA is ligated to linkers, amplified with polymerase chain reaction (PCR), and subsequently cloned into a plasmid vector. The clones are analyzed in dot blot hybridization for strain-specificity. We have applied a simple subtraction method (Straus D and Ausubel F 1990. Proc Natl Acad Sci USA 87:1889-1893) for our model strain, *Rhizobium galegae* HAMBI 1174, and isolated a 340 bp long DNA fragment which has proved to be strain-specific and can be used as a hybridization probe. The fragment has been sequenced and we have designed PCR primers for developing further identification methods. The primers are also strain-specific. DNA hybridization combined with the polymerase chain reaction technique should allow easy and rapid detection of bacteria from the environment.

A. NÉMETH, J. REICHARDT, J. FARKAS, I. BALOGH and É. ANDRÁSSY

Detection and characterization of bacteriocins formed by lactic acid bacteria

National Research Institute of Meat Industry, Department of Refrigeration and Animal Product Technology, and Chair of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary

Recently, isolation of bacteriocin-forming lactic acid bacteria have been reported by a number of authors. Some of the bacteriocins inhibit only several bacterial strains, others larger groups of microorganisms. Using the agar drop method and the agar diffusion method, we detected the listeria-inhibiting capacity in two bacteriocin-forming strains (*Leuconostoc gelidum* and *Lactobacillus sake* Lb 706), received from culture collections abroad and a strain (*Lactobacillus helveticus*) that we assumed to be bacteriocinogenic. The titre of bacteriocin activity on *Listeria monocytogenes* was 1:16, 1:32 and 1:8 in case of *Lactobacillus sake* Lb 706, *Leuconostoc gelidum* and *Lactobacillus helveticus*, respectively.

Both of the *Lactobacillus* strains under study were subjected to plasmid analysis. In *Lactobacillus sake* Lb 706 the single plasmid, already described in the literature, was detected, whereas from *Lactobacillus helveticus* six plasmids were isolated. The listeria-inhibiting effect of the bacteriocin formed by the two strains received from abroad was determined in liquid culture and a sterile foodstuff model as well.

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THE STRUCTURE AND FUNCTION OF THE FIRST COMPONENT OF COMPLEMENT: GENETIC ENGINEERING APPROACH (A REVIEW)

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The availability of cDNA and genomic clones for the subcomponents of C1, as well as the recognition of the modular organization of serine-proteases have opened up exciting new possibilities for approaching structural problems. In this review the latest achievements of combined protein engineering, functional and structural studies are summarized. The concept of this research is to construct deletion, point and hybrid mutants of the highly homologous C1r and C1s subcomponents, to reveal the functional role of individual modules, map the interaction sites between subcomponents of the C1 complex and refine the structural model of C1.

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The first prerequisite of such an approach was the expression of the subcomponents in a eukaryotic system, in biologically active form. This was followed by expression of various mutants.

Autographa californica nuclear polyhedrosis virus was used as vector to express human C1r and C1s in *Spodoptera frugiperda* cell culture and in lepidopteran larvae. The yield of expression was high enough to isolate recombinant subcomponents for structural and functional studies. Recombinant viruses containing the A-, B- and C-chains of C1q were also constructed.

The insect cells are able to β -hydroxylate the Asn residue of the EGF domain in the C1r but with a low efficiency. It is clear now, that this post-translational modification does not play a role in the Ca^{2+} dependent C1r-C1s interaction.

The results with deletion mutants of C1r show that both, domain I, and II are absolutely necessary for the tetramer formation and both have regulatory role in the autoactivation.

The C1s α R hybrid does not dimerize in presence of Ca^{2+} , however it can form a tetramer with C1I $_2$ that can bind to C1q. This observation indicates that the function of the C1s α part in the hybrid is modulated by the C1r part (γ B) of the molecule.

The C1Rs hybrid behaves like C1r, providing haemolytically active C1 with C1q and C1s. This observations shows that the regulatory domains determine the high functional specificity of the serine-protease subcomponents of C1.

In order to control the autoactivation process point mutant cDNAs were constructed by altering the Arg-Ile bond in the catalytic domain of the C1r. The Gln-Ile construction is a stable zymogen while the Arg-Phe mutant has a lower rate of autoactivation.

Introduction

Complement is a complex multicomponent system having many similarities with blood coagulation and fibrinolysis. The classical pathway of complement activation is initiated by the reaction of the first component, C1, with aggregated antibody. The first component is a heteropentamer of three subcomponents: one C1q, and two C1r and C1s. One specific inhibitor protein, C1-inhibitor, regulates the activation and function of the C1 complex. Extensive physical and biochemical studies have contributed substantially to our knowledge about the structures of the subcomponents and their assembly into C1 complex. Structural and functional models were suggested for the C1 complex [1-3]. These models are not free of speculative elements, still causing controversy as to the precise mode of interactions between the subcomponents, and the mechanism of activation upon binding to immune complexes. It is still not clear how biological function of C1 relates to its structure. The emerging protein engineering techniques and the concept of modular organization of extracellular enzymes opened up a new approach to study the structure and function of C1. The cDNA and genomic clones are now available for the subcomponents and C1-inhibitor. Successful expression of the recombinant glycoproteins in a eukaryotic expression system [4, 5] was the first step on the way to construct and produce mutants to carry out detailed studies on the protein-protein interactions involved in the assembly and function of C1.

Although we can draw a coherent overall picture of C1 structure and activation, there are still many mysteries about C1 that need to be elucidated. First of all the respective binding sites on the subcomponents are to be localized and mapped, the nature of the activation signal should be revealed at molecular level, the interaction with C1-inhibitor is also controversial. Sequence comparison, functional comparison among related modules that are comprised in the extracellular serine-protease subcomponents (C1r and C1s), combined with protein engineering experiments was used during the recent years in studying the outlined problems. C1r and C1s are both composed of six modules that were found in other extracellular proteases. The modular organization of the subcomponents makes the construction of deletion mutants possible without disturbing the protein folding. These two subcomponents are highly homologous in their sequences, but have different functions in the C1 complex. This high degree of similarity makes these proteins most appropriate for construction of hybrid mutants, i.e. to exchange domains between them and follow the changes in functional properties. The strategy of point mutant construction is based on preliminary information on the binding and functional sites (Ca^{2+} , autoactivation, dimerization, etc.). A systematically designed family of point, deletion and hybrid mutants proved to be a powerful tool in studying such a complex macromolecule like C1.

In this brief review the latest results concerning the structure of the subcomponents and the C1 complex, as well as the first results of a combined protein engineering approach are summarized. The successful expression of complex human glycoproteins of the complement system in insect cell culture and living insects using baculovirus vectors is of certain significance and together with the expression of various mutant subcomponents will enable us to refine the existing hypothetical C1 models for a better understanding of the mechanism of action of C1, this remarkable macromolecular complex of vertebrate defence mechanisms.

Structure of C1q

The six-stranded C1q subcomponent is the structural framework of the C1 complex. C1q is a large glycoprotein (460 kD) composed of 18 polypeptide chains (6A, 6B and 6C) each of which is approximately 225 amino acid residues long. The serum concentration of C1q is 80 $\mu\text{g/ml}$. The complete amino acid sequence of each of the three chains of human and mouse C1q is now known [6-9]. In each chain a short amino-terminal region of 2-11 amino acids is followed by a collagenous segment (repeating triplets of sequence Gly-Xaa-Yaa) of 78-84 amino acids, and a carboxy terminal noncollagenous region of 126-133 amino acids. Electronmicroscopy studies have shown C1q to be composed of six globular heads linked via six collagen-like stalks to a fibril-like central region [10]. According to the model proposed by Reid and Porter [11] one copy of each A-, B- and C-chains form a triplet, such that the three collagenous regions form a collagen triple helix, while the C-terminal regions of the three chains form a globular head. Each pair of A- and B-chains within a single triple helix is coupled by a disulfide bond, and a second disulfide bridge connects C-chains in adjacent triple helices to form a pair of helices and heads. Three such pairs associate noncovalently to form the complete molecule the familiar C1q bouquet (Fig. 1). The

collagenous triple helixes are bent at the middle owing to a discontinuity in the repeating Gly-Xaa-Yaa sequence in the A- and C-chains. The flexibility of this kink region may play an important role in the C1 activation [12, 13]. The human C1q A-, B- and C-chain genes contain a single intron and in each case the intron/exon boundary lies at the site of the interruption of the collagen structure in the A- and C-chains and at the equivalent region in the B-chain [6, 14]. The cDNA clones for all the three chains of human and mouse C1q have been isolated, as well as the genomic clones for the human A-, B- and C-chains [6–9, 14].

The three human genes have been shown to be aligned 5'–3' in the same orientation, in order A-C-B on the 24 kb stretch of DNA on chromosome 1p at 1p 34.1–1p 36.3 [6].

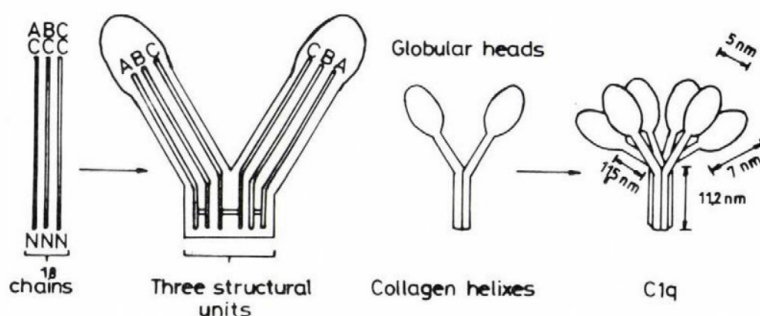


Fig. 1. Model of the C1q molecule. The molecule consists of 6 A, 6 B and 6 C chains. Two A-B and one C-C dimer associate to form a structural unit with two triple helices and two globular heads. Three such units form the complete C1q molecule

Recent structural studies and sequence comparisons revealed significant similarities between C1q and several non-complement proteins. The C-terminal globular-type sequences of the type VIII and type X collagens show strong similarity to the entire globular regions of the chains of C1q [15]. The conserved cluster of aromatic residues in this domain may be involved in C-terminal trimerization. Human precerebellin also shows a significant similarity (31.3% identity, 52.2% similarity) to the globular region of the B-chain of C1q [16]. The most interesting examples for structural similarity however are the four lectin-like molecules: mannan binding protein (MBP) [17], lung surfactant protein A and D (SP-A, SP-D) [18] and bovine conglutinin [19]. All of these molecules have collagen-like stalks attached to globular heads. The most striking example is the SP-A which is virtually indistinguishable from C1q in the electron microscope. The globular domains of these molecules belong to the Ca^{2+} -dependent C-type lectin consensus amino acid sequence and show no similarity in sequence to the globular C-terminal regions of C1q. MBP has been shown to mimic C1q functional activity by its ability to activate the $\text{C1r}_2\text{C1s}_2$ proenzyme complex after interaction with suitable carbohydrate ligands [20]. Studies of the mechanism of this activation process will provide useful comparison for C1, and are likely to clarify the general features of activation reactions.

C1q has at least three functions: (1) template for the assembly of C1, (2) binding to immune complexes via its globular heads, (3) transmitting the functional signal to C1r as a result of multivalent binding. The C1r₂C1s₂ tetramer is attached to the collageneous region of C1q. Although the precise sites of this interaction are unknown, we know that ionic bonds are involved. C1q binds to IgG or IgM via the globular head regions. Each head of C1q is likely to consist of three domains, with strong noncovalent association between them. Ultracentrifugation studies suggest an optimum of two IgG-binding sites per C1q head [21]. Charged residues may be involved in this interaction. A free cysteine at position 171 in each of the three chains may form a covalent bond between IgG and C1q [22].

Determination of the C1q residues involved in interactions with IgG, C1r₂C1s₂ and C1qR requires detailed structural information. The size and complexity of C1q make it a poor candidate for X-ray crystallography. Because of its three-chain based structure it has proven impossible to produce recombinant fragments in bacteria or yeast. Insect cells however seem to be appropriate to express the mouse C1q molecule in correctly folded and assembled form. Recombinant baculovirus vectors containing the A-, B- and C-chains of mouse C1q were constructed for this purpose. Smaller recombinant fragments (i.e. the globular head) can be suitable in size for crystallization or structure determination by NMR.

Structure of C1r and C1s

C1r and C1s are highly specific serine-proteases, which form the enzymatic part of the C1 complex. Both zymogens are single-chain glycoproteins (86–78 kD) containing 688 (C1r) and 673 (C1s) amino acid residues, respectively. There is a strong relationship between the two molecules: sequence comparison revealed 40% amino acid identity and conservation of all the cysteine residues [23]. The C1r and C1s genes are closely linked – probably as a result of a gene duplication – being within a 20 kb separation of each other on region p13 of human chromosome 12 [24]. Activation of the zymogens occurs through cleavage of a single Arg-Ile bond in the catalytic part of the molecules [25, 26]. The serum concentration of C1r and C1s is 50 µg/ml respectively. Isolated C1r is a dimer in the presence or absence of Ca²⁺ while the dimerization of the isolated C1s is Ca²⁺ dependent. When C1r₂ and C1s are mixed in the presence of Ca²⁺, they associate to form the C1s-C1r-C1r-C1s tetramer [27].

Both C1r and C1s have a mosaic like structure typical of plasma serine proteases. Since Leytus et al. [28], Tosi et al. [23] and Mackinnon et al. [29] have cloned and sequenced human C1r and C1s cDNA, useful information was deduced from sequence comparison and a protein engineering approach has also been launched [4] to reveal structural and functional details about C1. The principle of modular organization of some vertebrate proteins, particularly serine proteases [30] was a great help in deducing structural and functional conclusions from sequence data. Both C1r and C1s can be divided into six structural motifs (domains or modules), including two pairs of internal repeats (I/III and IV/V), a single copy of motif II and the trypsin-like serine-protease (SERP) modul (Fig. 2). Moduls I and III are homologous domains found first in C1r [28] and in C1s [23]. Bork [31] has identified related domains in four

developmentally regulated proteins, one of that is a Ca^{2+} dependent serine protease. The role of these domains is unknown, they may be involved in a function that is highly specific to C1r and C1s (for example: C1r-C1s interaction, $\text{C1r}_2\text{C1s}_2\text{-C1q}$ interaction). McAllister and Fothergill [32] have cloned and transformed into a yeast expression system [33] the N-terminal 100-residue repeat of C1s. High resolution NMR studies are underway to reveal the 3D structure of this motif.

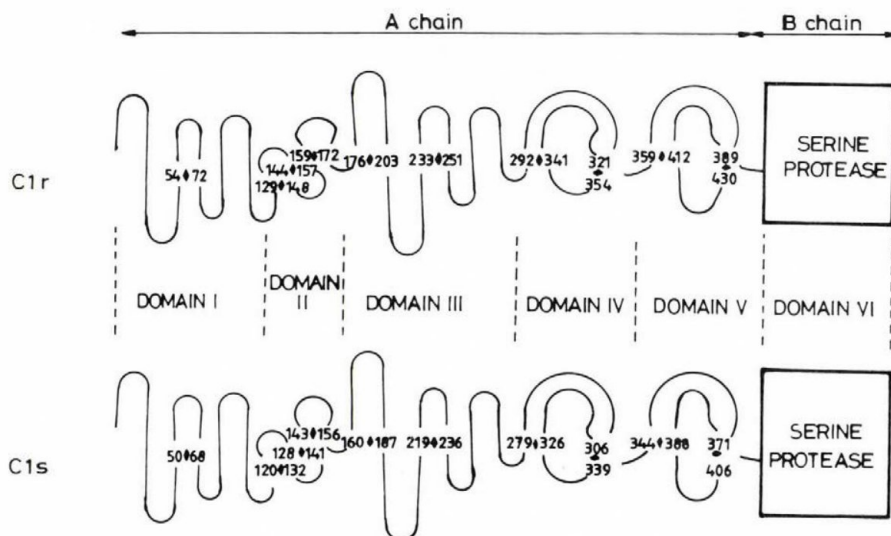


Fig. 2. Domain structure for C1r and C1s. Disulfide bridges are indicated (♦). (Partly adopted from Leytus et al. [28] and Fothergill et al. [69].)

Module II shows homology with epidermal growth factor (EGF), including six cysteine residues distributed in the pattern characteristic of EGF-like domains [23, 28]. In general such domains are exposed to the extracellular environment and participate in protein-protein interactions. This domain harbours an unusual amino acid, β -hydroxy asparagine in position 150 in C1r and in position 134 in C1s. The level of hydroxylation in native human C1r is 100% while in C1s is only 50% [34]. This module may play an essential role in Ca^{2+} binding and the $\text{C1r}_2\text{C1s}_2$ tetramer formation.

Motifs IV and V are 60 amino acid residue long ubiquitous sequence elements (short consensus repeat SCR or complement control protein CCP modul) found in multiple copies in various complement proteins and other proteins unrelated to the complement system [35, 36]. The 3D structure of this motif was determined by NMR [33]. This module consists of a double stranded antiparallel β -sheet with three disulfide bridges. These two modules are closely associated with the catalytic domain (B-chain)

and are involved in the C1r dimerization and probably in the interaction of C1s with C4 and C2.

The serine-protease domains are homologous to the trypsin family [37]. The most characteristic property of C1r and C1s proteases is their highly restricted specificity. C1r has only two natural substrates: C1r and C1s. C1r in the C1 complex can autoactivate itself. The activated C1r then cleaves the Arg-Ile bond in another zymogen C1r or C1s. Then C1s activates the next components of the complement cascade: C4 and C2, through proteolytic cleavage. The high specificity of C1r and C1s proteases can be explained by the mosaic structure of these proteins: the five noncatalytic domains determine the unique substrate specificity of the SERP domain in these molecules. The analysis of the genomic clones of C1r and C1s revealed the fact, that the protease domains are encoded as intronless domains [38]. In the case of other serine-proteases the zymogens are always encoded by multiple exons.

In the electron microscope C1r and C1s were visualized as dumb-bell-shaped molecules consisting of two globular domains connected by a rod like stretch [1]. Limited proteolysis experiments also yielded two type of compact globular domains: the N-terminal interaction region (α), containing motifs I, II and part of the motif III, and the catalytic domain (γ B), containing motifs IV, V and the SERP module at the C-terminal. The C1s α fragment can bind to the C1r₂ in presence of Ca²⁺ and this tetramer can bind to the C1q [39]. On the other hand C1r γ B fragment can dimerize and the dimer has autoactivation capacity and serine-protease activity [40]. The highly specific multifunctional C1r₂C1s₂ tetramer is the functional core of the C1 complex. Unfortunately little is known about the intramolecular mechanism of its activation. Deletion, replacement and modification of modules in these subcomponents offer a powerful tool in assigning specific functions to individual modules, and in revealing the mechanism of activation.

Structure of C1-inhibitor

C1-inhibitor is a highly glycosylated 104 kD plasma glycoprotein that belongs to the superfamily of serine protease inhibitors (serpins). C1-inhibitor inhibits activated C1r and C1s [41], as well as prevents spontaneous activation of unactivated C1 [42, 43]. It is also a critical regulatory component of the coagulation, fibrinolytic and kinin-releasing system. Other members of the serpin family include α_1 -antitrypsin, α_2 -antiplasmin, antithrombin III and plasminogen activator inhibitor types I and II. In serum, the concentration of C1-inhibitor is 137 μ g/ml, and the molar ratio of C1 to C1-inhibitor, approximately 1:7. Genetic deficiency of C1-inhibitor, resulting from either quantitative or structural alterations, is the cause for the disease hereditary angioedema [44].

Electron micrograph and neutron scattering experiments have revealed a molecule shaped rather like a match, with a bulbous head and a rod-like tail [45, 46]. The primary structure of human C1-inhibitor was determined by peptide and DNA sequencing [47]. The single chain polypeptide is 478 amino acid residues long, 49% of the total molecular mass of C1-inhibitor is added as a result of posttranslational glycosylation. Most of the carbohydrate chains (approx. 17) are located at the amino

terminal tail of the molecule (Fig. 3). C1-inhibitor is the only known inhibitor of C1r and C1s. Activated C1, C1r₂C1s₂, C1s and C1r₂ are all rapidly inactivated by formation of tight complexes with C1-inhibitor. Complex formation is characterized by the irreversible cleavage of the peptide bond at Arg444-Thr445 in the reactive centre of the inhibitor [48]. Conformational changes after cleavage lead to a much more stable protein structure [49]. The complex resists boiling in SDS, but it can be dissociated with hydroxylamine. These facts suggest that a covalent ester bond has been formed between the reactive center of the inhibitor and the catalytic domains of activated C1r or C1s. With C1r₂C1s₂ the complex probably has the form C1-inh-C1s-C1r-C1-inh [50]. The significance of the rod like domain of C1-inhibitor is not fully understood. The C-terminal head contains the reactive center of the inhibitor. One possible function for the heavily glycosylated N-terminal domain of C1-inhibitor is that this blocks the accessibility of the interaction region (domain I to V) in C1r and C1s after the serpin domain of C1-inhibitor has bound to the serine-protease domain [46]. Expression of truncated and aglycosylated recombinant C1-inhibitor might facilitate our understanding of the regulatory role of this glycoprotein of the serpin family.

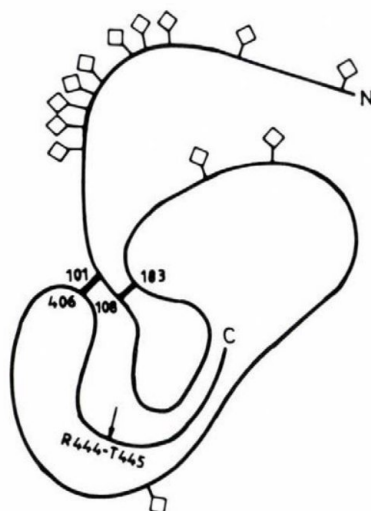


Fig. 3. Schematic representation of C1-inhibitor. The disulfide bridges are indicated by bars. The diamonds show the known glycosylation sites. The arrow indicates the Arg444-Thr445 dipeptide in the reactive center of the inhibitor. (Adapted from Bock et al. [47])

Structure of the C1 complex

The C1 supramolecular complex consists of one molecule C1q and one C1s-C1r-C1r-C1s tetramer. Unfortunately we have only limited information concerning the C1 structure. Electron micrographs of the chemically crosslinked C1 complex show the tetramer as a compact mass centrally located on the C1q stems, between the globular

heads and the stalk [51]. Based on this observation, on electron micrograph of the isolated $C1r_2C1s_2$, on the domain structure of $C1r$ and $C1s$ as well as on symmetry considerations functional models of $C1$ has been elaborated during the last few years [1-3]. The common feature of these models is that the $C1r_2C1s_2$ tetramer is folded around the collageneous $C1q$ arms, so that the catalytic subunits of $C1r$ and $C1s$ can contact each other upon activation (Fig. 4). According to this model the $C11(\gamma\beta)_2$ part of the tetramer is located inside the cone defined by the $C1q$ stems, while the α interaction fragments lying outside the collageneous arms of $C1q$. The $C1r_2C1s_2$ tetramer adopts a compact, 8-shaped conformation in $C1$ complex, so that all four of the proenzyme domains are brought together, permitting access of the γB domain of $C1r$, located in the middle of the tetramer, to the terminally located γB domain of $C1s$. Several lines of evidence indicate that the N-terminal α regions of both $C1r$ and $C1s$ are responsible for the binding of the tetramer to the collagen-like $C1q$ arms [39]. Since ionic bonds are involved in this interaction, the $C1$ complex readily dissociates with increasing ionic strength [52]. The side chains of both components participating in this interaction remain to be identified. Activation occurs, when $C1$ binds to an asymmetric cluster of Ig-s on the immune complex. Because of the semi-flexibility of the kink region of $C1q$, the cone formed by the spreading $C1q$ arm will be distorted [13]. This distortion might be the activation signal that induces a conformational change converting the $C1r$ proenzyme into an enzyme. An alternate mechanism of activation involves rotation of $C1q$ arms upon binding to the immune complex [12]. Once the Arg-Ile bond has been cleaved in the $C1s$, the tetramer adopts a more relaxed conformation, which permits the catalytic domain of $C1s$ to protrude outside the $C1q$ cone. Following activation of $C1$, the complement cascade is sustained through the proteolytic action of $C1s$. Loosening of the tetramer would increase accessibility of not only the activated $C1s$ but also for the $C1r$ to $C1$ -inhibitor. Once $C1$ -inhibitor has bound to the activated components they readily dissociate from the $C1q$ as a $C1$ -inhibitor- $C1r$ - $C1s$ - $C1$ -inhibitor complex.

Although the symmetrical models for $C1$ are in good agreement with the electron microscopic data and the domain structure of the subcomponents, asymmetrical models also exist. In these models [53, 54] the $C1r_2C1s_2$ tetramer is placed outside the six $C1q$ stalks. Although these models can explain how the tetramer can readily dissociate from $C1q$ after dilution of sera or increasing the salt concentration, and the accessibility of the SERP domains of the activated tetramer to $C1$ -inhibitor, a major disadvantage of these models however is, that they are not consistent with electron micrographs showing the major part of the tetramer being located inside the $C1q$ cone. It should be emphasized here, that the available $C1$ models are rather speculative due to the lack of direct information about the 3D structures of the subcomponents, and consequently the complex itself.

Protein engineering studies

In order to provide a template for future protein engineering studies we have sought to express the cDNA of human $C1r$ and $C1s$ subcomponents in a eucaryotic host-vector system. The more commonly used prokaryotic expression systems seemed

unsuitable because of posttranslational modification and folding problems. It has been shown that the baculovirus-insect cell system can express foreign gene products including complex glycoproteins. For most recent reviews and complete list of expressed proteins see the publications of O'Reilly et al. [55] and Vlak et al. [56]. The insect cells are able to process the newly synthesized eucaryotic proteins, therefore biologically active gene products can be obtained. We have placed the full-length cDNA of human C1r and C1s under the control of the strong polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and investigated its expression in insect cells (Sf9; High5) and checked the biological activity of the products [4, 5, 57, 58].

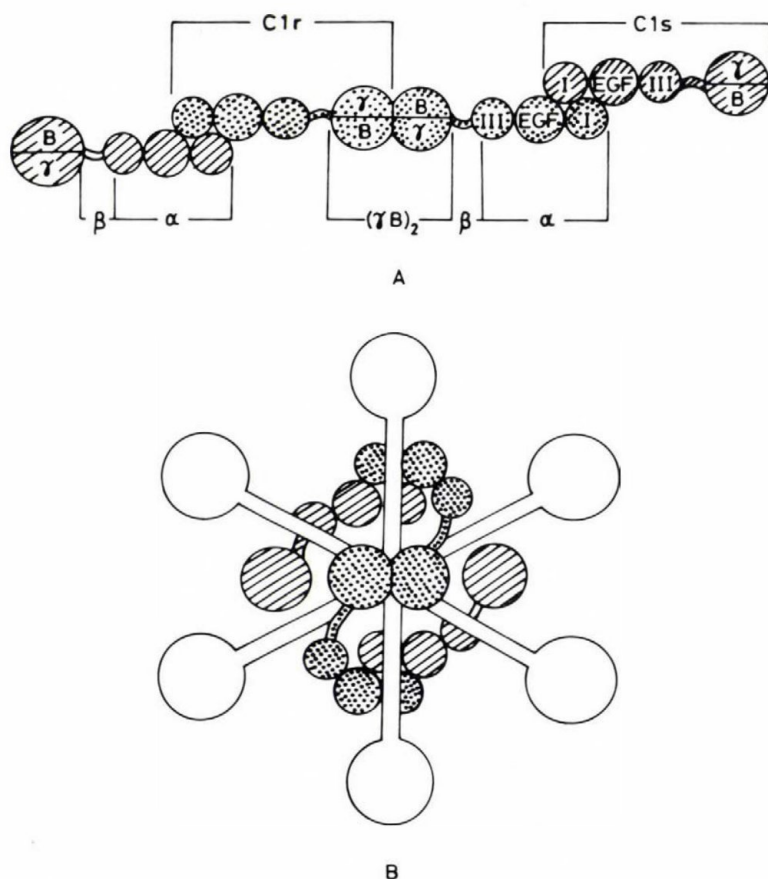


Fig. 4. Functional model of the C1 complex. (A) Model of the C1r₂s₂ tetramer. (B) Model of the C1 complex. The view is into the cage of C1q, showing the tetramer folded around the stems. The C1r(γB)₂ is located in the center of the cone while the C1rα-C1sα region of the tetramer is wound outside the C1q arms. The C1s catalytic domains can contact either with the C1r(γB)₂ (inside the cone) or with C4 and C2 (outside the cone)

C1r and C1s cDNA-containing recombinant viruses. The recombinant virus vector for C1r expression contains the entire coding region of 2115 nucleotide pairs and in addition a noncoding region of 63 base pairs at the 5' end, and a noncoding region of 8 base pairs at the 3' end, placed downstream of the strong baculovirus polyhedrin promoter. The recombinant virus for C1s contains the 2067-bp coding region and the 293-bp 3'-untranslated region. To avoid possible interference on the expression, the 5'-untranslated sequence was excised in the case of C1s. We have to note here, that in the case of C1r, the elimination of the 5' noncoding region, did not affect the yield of expression.

Expression of human C1r and C1s in insect cells. Insect cells (Sf9, High5) were infected with the recombinant baculoviruses, and three days post infection the cell culture supernatant was collected and analyzed for the presence of recombinant proteins. The recombinant proteins were isolated from the cell culture medium by ion exchange chromatography (DEAE-Sephacel for C1s) [5] and Superose 12 gel filtration FPLC column for C1r as well as by affinity chromatography (C1r) [58]. The homogeneity and the molecular mass of the proteins were checked by SDS-PAGE and Western blot.

Biological activity. The biological activity of the recombinant components was tested by using the haemolytic assay [59]. C1 was reconstituted from serum C1q and recombinant tetramer. In this way haemolytically active C1 was generated. We believe this to be the first report of a sophisticated heteropentamer zymogen produced in functional form by the baculovirus-insect cell expression system.

Posttranslational modifications of the recombinant proteins. (i) *Signal peptide cleavage.* Because both, recombinant C1r and recombinant C1s were secreted into the medium it is obvious that insect cells can recognize the leader sequences coded by the human cDNA, and process these proteins. The processing includes the cleavage of N-terminal signal peptides (15 amino acids) by the insect signal peptidase. The sequence analysis of the recombinant C1s showed that its N-terminal is identical to that of the human serum C1s [5].

(ii) *Glycosylation.* On the SDS-PAGE the native and recombinant form of C1r and C1s migrated with a small but significant difference in mobility indicating that the carbohydrate content of the proteins may be different. This difference was more significant in the case of the A chain of recombinant C1s while the B chain had the same mobility as the serum C1s B chain [5]. The two glycosylation sites of C1s are both located on the A chain. It has been shown in many cases that insect cells can recognize the N-glycosylation sites of higher eucaryotic proteins, but insect cells usually do not perform complex type glycosylation [60]. In the case of the recombinant proteins the incompletely processed N-linked carbohydrate chains lack at least, the terminal sialic acid residues. This difference however does not reflect in the biological activity of the recombinant C1r and C1s.

(iii) *β -hydroxylation of Asn in the EGF domain.* The posttranslational β -hydroxylation of specific aspartyl and asparaginyl residues has been shown to occur stereospecifically within certain epidermal growth factor-like (EGF) modules of several mammalian proteins [61, 62]. Both C1r and C1s has a potential site for this type of modification in the EGF domain (Asn 150 in C1r, Asn 134 in C1s). The level of hydroxylation in native human C1r is 100%, while in C1s is only 50%. It was

suggested that by analogy with similarly modified amino acids in other serum proteins, β -hydroxyasparagine is involved in calcium binding [2]. Reconstitution experiments, and haemolytic assay with recombinant C1r and C1s [4, 5] do not support this view. Although insect cells have the potential for aspartyl/asparaginyl hydroxylation, as it was shown by us for the first time [58], their efficiency of hydroxylation is low. Only 10% of recombinant C1r is hydroxylated, while we could not detect β -hydroxylation in the case of C1s [5]. The lack of β -hydroxyasparagine does not impair neither the calcium dependent assembly of the subcomponents [4] nor the haemolytic activity of the C1 complex.

The discovery of the potential of invertebrate aspartyl/asparaginyl hydroxylation is an important byproduct of our complement expression studies in baculovirus-insect cell system. Although the EGF modules have been shown to bind calcium [63, 64] and to mediate protein-protein interactions, the role of hydroxylation has not been revealed. Our studies have demonstrated that like EGF modules themselves, the corresponding hydroxylase activity is also present in insects. This fact indicates that EGF module-dependent posttranslational modifications may be important in regulating EGF module mediated protein-protein interactions. Although this function has not been yet revealed.

Increasing the yield of C1r expression. In order to get higher amount of recombinant protein we modified the expression system. At DNA level we made C1r cDNA constructions using different transfer vectors and the 5' noncoding region of the cDNA was removed. We could increase the yield from 1.3 to 3 $\mu\text{g/ml}$ supernatant using vectors which have the complete polyhedrin noncoding region (pAcYML, pVL941). However the 5' noncoding region of the human cDNA did not influence the yield of expression.

In cell cultures we applied insect molting hormones to stimulate the protein synthesis. 20-hydroxyecdysone proved to be the most efficient, producing a three fold increase in the level of recombinant protein secreted into the medium. This effect was also confirmed by tracing the L-(^{35}S) methionine incorporation into the gene product. Makisterone was also effective in stimulation, while ecdysone proved to be ineffective [57].

Although most expression work has thus far been conducted in cell cultures, larval production can be an extremely useful alternative to expression in cell culture, for generating large quantities of recombinant proteins. We used our recombinant viruses to infect the larvae of two lepidopteran species, *Mamestra brassicae* and *Manduca sexta*. We used different methods for infections and optimized the time of protein harvesting from the insects haemolymph. In this way we can produce large amounts of biologically active recombinant protein at significantly reduced cost.

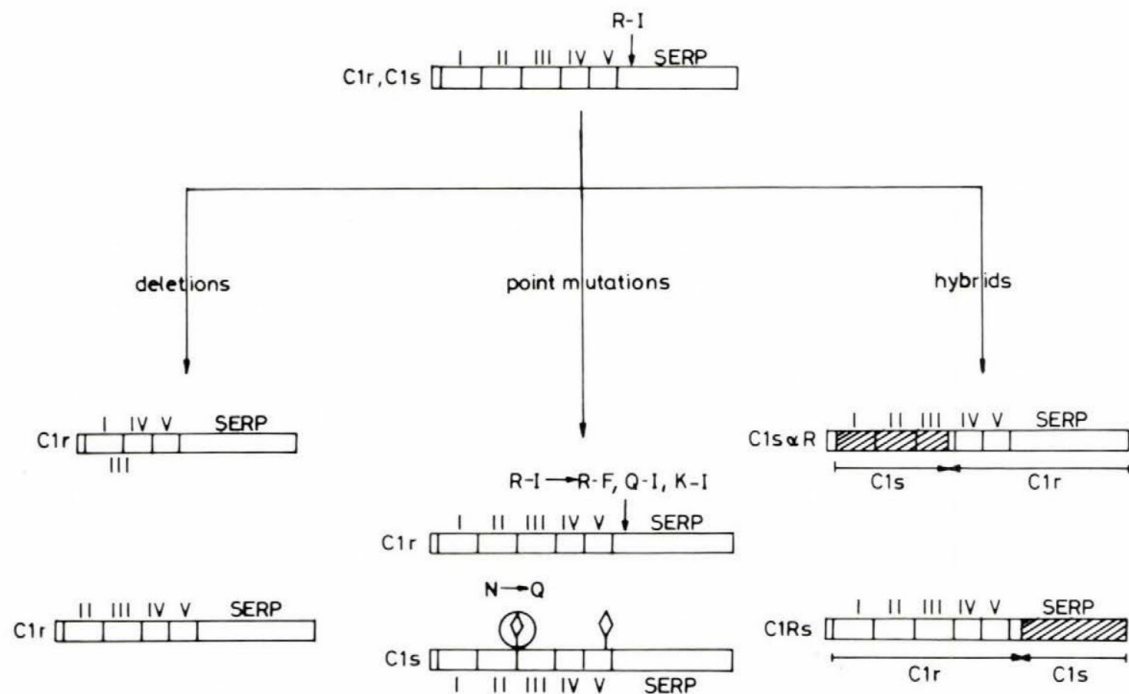


Fig. 5. The strategy of mutation experiments. Diamonds indicate the Asn-linked carbohydrates of C1s. The arrow shows the Arg-Ile bond which is cleaved under activation

Mutagenesis

The expression of intact C1r and C1s in biologically active form has opened the way for protein engineering studies. Our strategy, to reveal the structure and mechanism of activation of C1 at the molecular level, is based on the combination of genetic, physical and functional studies. Since we do not have the 3D structure of the subcomponents, we could not perform "real" protein engineering; i.e. changing definite amino acid residues that are important in protein function according to the 3D model. All the structural information we have – as described in the earlier sections – originated from electron microscopy, domain prediction from amino acid sequence, and speculative model building of C1. The approach we took involved the deletion or changing of larger parts – i.e. domains – of the C1r and C1s molecules. Our aim was to reveal the role of the individual domains in the structure and activation of C1. In addition we made several point mutants by changing the activation dipeptide Arg-Ile of C1r and a glycosylation site of C1s. The scheme of our approach is shown in Fig. 5.

Deletion mutant 1. We have constructed two deletion mutant C1r cDNAs. In both cases the deletion concerns the N-terminal noncatalytic region of C1r. In our first deletion mutant C1r cDNA construction the peptide Ile61-Gln239 is deleted. We used the high degree of homology between the first two internal repeats (domain I and III) of C1r for constructing this mutant. Both domain I and III were cut at equivalent positions and religated after deletion of the fragment. Folding and reading frame considerations were taken into account. The new molecule lacks the entire EGF domain, and one of the internal repeats. In other words it contains a hybrid domain with the first half of domain I and second half of domain III, and the γ B fragment. We made the recombinant virus and infected Sf9 cells. The mutant protein was expressed and secreted by the cells into the medium at high concentration (3 μ g/ml). The good yield of the expression indicated, that there were no folding or processing problems.

The mutant protein could be detected as a dimer and showed proteolytic activity as measured on synthetic substrates. We could not detect any interactions with C1s and C1q. These results confirm that this region near to the N-terminus is essential for binding to the other subcomponents. The selfactivation of the zymogen was highly accelerated, compared to the wild type rC1r. This points to an important function of the N-terminal noncatalytic region of C1r: it stabilizes the zymogen form of the serine-protease domain.

Deletion mutant 2. In order to reveal the role of domain I in C1r we have deleted a sequence between the leader and domain II from the cDNA of C1r (Cys 4-Pro82). The mutant protein was expressed and secreted into the medium by Sf9 cells. Dimerization and proteolytic activity on synthetic substrate were checked. We know that the Ca^{2+} dependent interaction between C1r and C1s – which is essential for the $\text{C1r}_2\text{C1s}_2$ tetramer formation – takes place through the N-terminal α -region of the molecules. There are two different models however concerning this interaction. Tosi et al. [23] stress the significance of domain II, which might be responsible for Ca^{2+} binding and C1r-C1s interaction, while Illy et al. [65] claim that the structural determinant of C1s required for Ca^{2+} binding and Ca^{2+} dependent protein-protein interactions are contributed by both domain I and II (Fig. 6). In order to test these models we checked the tetramer formation using C1s and our deletion mutant. We

could not detect any tetramer. This result supported the model proposed by Illy et al. [65], suggesting that ionic bonds – with or without a Ca^{2+} bridge – between motif I of C1r and C1s contribute significantly to the C1r-C1s binding. One additional important feature of the domain I deletion mutant – and the other deletion mutant as well – is the accelerated ability of autoactivation. In contrast to the recombinant wild type C1r [4] the mutant is completely activated in the cell culture supernatant, i.e. no zymogen form can be detected by Western blot, even in the presence of calcium. This observation reveals the regulatory role of the first domain in the process of autoactivation and in the modulation of proteolytic activity in general. This effect also calls the attention to the existence of interdomain signal transmitted from the α -region to the γB catalytic region in C1r [66].

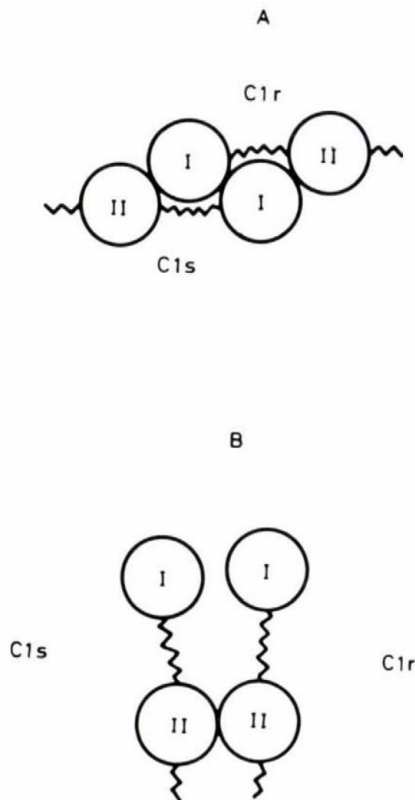


Fig. 6. Schematic representation of two different suggestions for the C1r-C1s interaction. (Only domain I and domain II are indicated.) (A) Interaction through the first and second domains. (Adopted from Illy et al. [65]) (B) Interaction through the second domains. (Adapted from Tosi et al. [23])

Hybrids. The molecular structure of the two subcomponents and the high degree of homology between them offered the possibility to construct hybrid molecules. Two mutant cDNAs of this type has been constructed and one of them has been expressed in the insect cells.

Busby and Ingham [39] showed, that the N-terminal α -fragment of C1s – which was generated by plasmin digestion of intact C1s and comprises motif I, II and part of motif III – can self associate in the presence of Ca^{2+} or bind to the C1r₂ dimer. While activated C1r₂ alone cannot bind to the C1q, the binding affinity of the C1s α -C1r-C1r-C1s α tetramer to C1q is equal to that of the C1s-C1r-C1r-C1s tetramer. This experiment provided evidence that all of the Ca^{2+} dependent interactions of human C1s with itself and with other subcomponents of C1 are mediated by the C1s α fragment. It was not clear however, whether C1s α carries all the contact sites necessary for C1q binding or it induces a conformational change in C1r that enhances its affinity for C1q. To answer these questions and to characterize further the role of the C1s α fragment we made a hybrid molecule, comprising the N-terminal motifs I, II and a large part of III from C1s and the remaining part of motif III and motifs IV, V and SERP from C1r. This C1s α R hybrid contains the N-terminus of C1s to Asp255 and the C1r sequence from Leu268 to Asp688. This particular ligation is based on functional, folding and homology considerations. We expected this mutant to self associate as C1r, and as C1s in the presence of Ca^{2+} , and to make a C1s α R₂C1s₂ tetramer, which tetramer might bind to C1q. Surprisingly however, this hybrid cannot dimerize neither as C1r through its γ B region nor as a C1s through its α -region in the presence of Ca^{2+} , but it can form a tetramer with C1r₂, and this C1s α R-C1r-C1r-C1s α R tetramer can bind to C1q. These results provide proof that in both C1r and C1s the noncatalytic N-terminal region and the catalytic γ B region are not independent. In the C1s α R hybrid the C1s α fragment behaves differently compared to the intact C1s or C1s α alone. On the other hand the C1r γ B part of the molecule shows some features characteristic of C1s, it cannot dimerize, but can activate itself, as checked by Western blot. The binding of the hybrid to C1r₂ is significantly weaker than that of the native C1s. C1s α R-C1r-C1r-C1s α R tetramer binds to C1q, but the product does not show haemolytic activity. The C1s α part was not enough to change the substrate specificity of the γ B region, i.e. C4 and C2 cannot be cleaved by the hybrid. Measurements to check C1-inhibitor binding and protease activity on synthetic substrates are in progress. The autoactivation of the hybrid is also an unexpected feature, because the autoactivation has thus far been thought as a function of the γ B dimer of C1r. These results did justify our approach of using domain-domain interchange in our protein engineering studies. The expressed recombinant hybrid has preserved some functions of the parent proteins and some functions were impaired. One obvious deduction is that C1 is a sensitive “well designed” structure and even homologous replacement of certain motifs in the regulatory part do impair biological function.

The second hybrid cDNA was constructed through joining the A-chain of C1r (motifs I, II, III, IV, V) and the B-chain (SERP) of C1s. This mutant will show us how the noncatalytic A-chain can change the substrate specificity of the catalytic B-chain. We are particularly interested in the role of the SCR domains in the C1r γ B dimerization, and autoactivation. The recombinant protein has been expressed. This

C1Rs hybrid autoactivates, and behaves like C1r in complex formation, providing haemolytically active C1 with C1q and C1s. These observations show that the regulatory domains determine the high functional specificity of the serine-protease subcomponents of C1. Further experiments are in progress on this mutant.

Point mutants. The activation of the zymogen C1r and C1s occurs through the cleavage of the Arg-Ile bond in their serine-protease domain [25, 26]. In the case of C1r this process is autocatalytic while zymogen C1s is cleaved by activated C1r. In order to influence the activation process we made three point mutant C1r cDNA constructions. The Arg-Ile bond is a rather conservative one among the serine-proteases: the activation of chymotrypsinogen and prothrombin also requires the cleavage of Arg-Ile bond. In the case of trypsin, which is homologous to the SERP domain of C1r and C1s the corresponding dipeptide is Lys-Ile.

In order to prevent the activation we changed the Arg446-Ile447 to Gln446-Ile447 in C1r. This dipeptide cannot be split by any serine-protease. The isomorphic replacement of Arg to Gln should not disturb the folding of the protein [67]. We have two aims by making this mutant. First, we intend to study the intact zymogen C1r molecule. Because of the spontaneous autoactivation of C1r it has proven difficult to isolate pure C1r proenzyme even in the presence of protease inhibitors. The C1r zymogen has a central role in C1 activation. Dodds et al. [68] suggest that after binding to immune complex the C1r acquires an intermediary form: "an unsplit form of subcomponent C1r which has a catalytic site able to cause the hydrolysis of C1r". To test this hypothesis we expressed the mutant in insect cells. It proved to be folded, it could dimerize and form tetramer with wild type C1s in the presence of calcium. This tetramer associated with C1q to form C1. Functional studies with C1 assembled from a 1:1 mixture of wild type and mutant C1r (Gln-Ile) are in progress to reveal whether the stable zymogen can activate C1r. Our other aim with this mutant is the crystallization for X-ray structure analysis. The crystallization of C1r has been attempted in several laboratories and has failed so far. The reason could be the autoactivation. During incubation a heterogeneous population arises containing intact and activated molecules with different conformation and flexibility. Therefore the stable zymogen could be a better candidate for crystallization.

An other point mutant that satisfied the folding criteria was the Arg446-Phe447 variant. Our aim with this mutant was to obtain a C1r zymogen with different rate of autoactivation. This mutant was expressed and purified. The mutation stabilizes the zymogen as judged by Western blot, but it does not affect the biological activity, since the rate limiting step in the haemolytic assay is slower than the autoactivation of this point mutant.

The third construction in this series is the Lys-Ile mutant. We would like to test the specificity of the autoactivation step using this mutant. Because the SERP domain of C1r is a trypsin-like serine-protease there is a possibility that this bond will be cleaved during autoactivation. If this mutant cannot activate itself, it will be activatable with trypsin. The expression of this mutant is in progress.

Our C1s mutant is the first member of an other mutant series. We would like to produce proteins without carbohydrate for crystallization. It was mentioned in the previous section, that the glycosylation of protein in insect cells are different from the glycosylation in mammalian cells. However this difference in glycosylation does not have any effect on the biological function of these molecules. C1s has two

glycosylation sites both located on the A-chain. One of them has been removed by converting Asn159 to Gln159 [5]. This mutant was efficiently secreted and formed haemolytically active C1 with C1r and C1q. These results provide further proof that carbohydrate is not required for folding or export of the protein in insect cells, nor is it essential for reassembly or C1 function.

Perspectives

Encouraged by these results we intend to continue the protein engineering of human C1r and C1s. Besides the catalytic subcomponents of C1 we will extend our work to the noncatalytic subcomponents: i.e. C1q and C1-inhibitor. Recently we have cloned the A-, B-, and C-chains of mouse C1q into the baculovirus vector and we attempt to co-express all the three chains at the same cell. If the assembly of C1q from the chains occurs, it will be the most sophisticated recombinant protein ever expressed in insect cells. If we succeed, we will extend our mutagenesis approach to C1q. We are particularly interested in altering the kink region. We are going to express recombinant human C1-inhibitor, as well. Protein engineering studies could help us in revealing the function of the heavily glycosylated amino terminal domain of C1-inhibitor. One of our major goal is to express proteins or protein fragments (i.e. C1q head, C1 α , C1 γ B) suitable for crystallization and X-ray structure determination. Based on these results we can envisage further specific experiments.

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Abbreviations

- AcMNPV *Autographa californica* nuclear polyhedrosis virus
- bp base pairs
- CCP complement control protein
- C1 α R hybrid molecule containing the $\alpha\beta$ region of C1s and the γ B region of C1r
- C1Rs hybrid molecule containing the A chain of C1r and the B chain of C1s
- EGF epidermal growth factor
- High5 *Trichoplusia ni* cell line
- MBP mannan binding protein
- SCR short consensus repeat
- SERP serine-protease
- Sf9 *Spodoptera frugiperda* 9 cell line
- SP-A lung surfactant protein A
- SP-D lung surfactant protein D

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TRANSFER AND ESTABLISHMENT OF DNA IN *STREPTOMYCES*

(A BRIEF REVIEW)

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Based on endogenous *Streptomyces* plasmids we have constructed various multiple purpose vectors for cloning in streptomycetes. Since replication of the *S. ghanaensis* plasmid pSG5 is inherently temperature-sensitive, pGM-vectors derived from pSG5 can be used for gene disruption/replacement and mutational cloning. The 1.6-kb minimal replication region of pSG5 encodes only one polypeptide, an initiation protein (Rep) for single stranded DNA-replication. Different internal fragments of sequenced genes of the Ptt-biosynthetic pathway were cloned into pGM-vectors to study both, the function of the biosynthetic genes and recombination in *Streptomyces*. The observed recombination frequencies were very high, up to 80% of the cells carried single or multiple copies of the plasmid integrated into the chromosome. Replacement experiments revealed that the frequency of marker exchange and plasmid integration, respectively, lies in the same order of magnitude. The region of homology which is required for homologous recombination could at least be reduced to 200 bp.

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Introduction

Bacteria of the group of *Actinomycetales*, in particular *Streptomyces* are known for their ability to synthesize various compounds which are produced on an industrial scale. The filamentous soil bacteria make secondary metabolites, such as antibiotics and other pharmacologically active molecules, as well as a great variety of extracellular enzymes such as amylases, cellulases, proteases and nucleases [1]. Synthesis of all these compounds is correlated with cellular differentiation [2]. The life cycle begins with the germination of a spore which gives rise to a multicellular substrate mycelium of branching hyphae. After depletion of the nutrients aerial mycelium is formed, followed by the fragmentation of the aerial hyphae into spore chain [3].

The regulation of the physiological and morphological differentiation is very complex and, at present, only partially understood.

In addition to their significance as industrial microorganisms and as model organisms for studying differentiation, streptomycetes play an important role in microbial ecology. *Streptomyces* strains are found in almost all kinds of soil and are involved in the degradation of high-molecular-weight compounds.

Therefore, the development of gene transfer systems for cloning experiments and the investigation of natural gene transfer in *Streptomyces* are prerequisites for both biotechnological application and understanding the biology of these bacteria.

Results and discussion

1. DNA transfer systems for *Streptomyces*

Gene transfer techniques using phage or plasmid vectors have been developed since the early eighties [4, 5]. Originally, vectors were constructed for the isolation and expression of *Streptomyces* genes.

In the meantime, novel vectors were constructed which can be used to analyse the function of genes by reverse cloning [6]. For that, certain DNA-fragments have to be cloned on suitable vectors, introduced into *Streptomyces* and stably integrated in the genome to generate defined mutations.

We have concentrated on the development of such systems and we have used them to study the transfer of DNA into different *Streptomyces* strains and the establishment of the transferred DNA. The systems are based on a temperature-sensitive (ts) *Streptomyces* plasmid and on non-replicative plasmids.

1.1 Characterization of the temperature-sensitive *Streptomyces* plasmid pSG5

The plasmid pSG5 was isolated from *Streptomyces ghanaensis* 5/1B [7]. It has a size of 12.7 kb and a copy number of approximately 50 per chromosome [8]. Since the host range of pSG5 comprises all *Streptomyces* strains tested so far, it is a suitable replicon for vector construction. Its minimal replicon was localized on an 1.6 kb fragment by Tn5 mutagenesis and by subcloning [9].

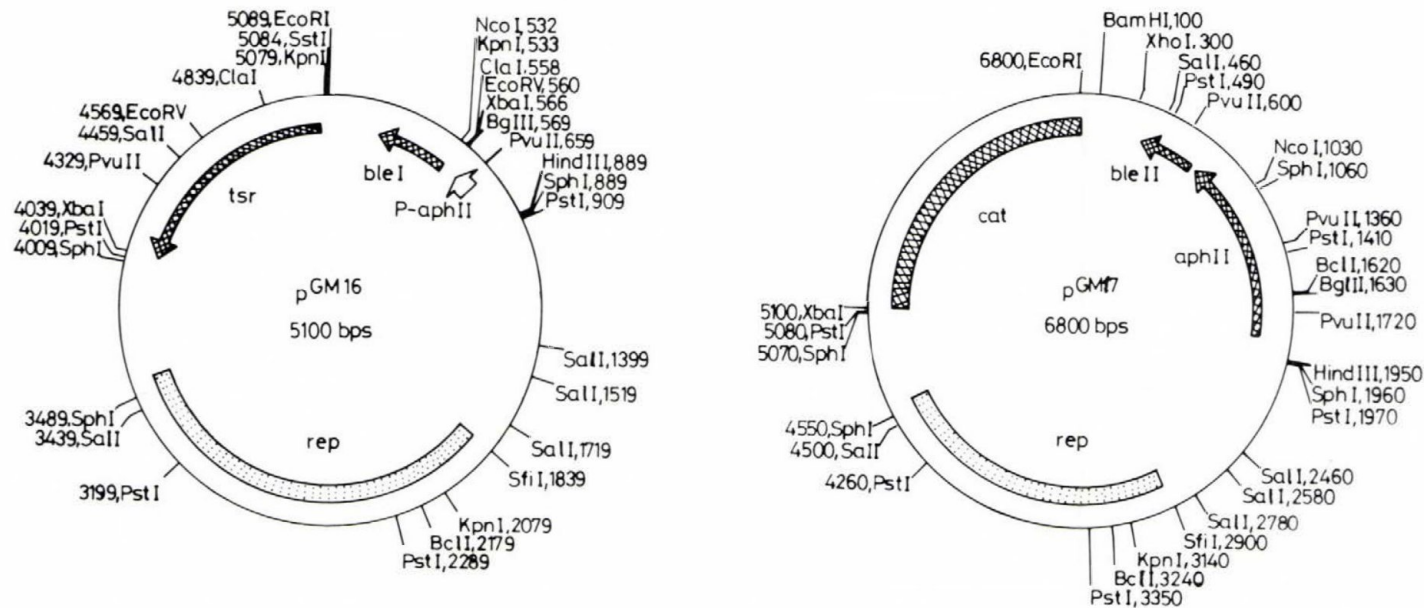


Fig. 1. Restriction maps of the *Streptomyces* vectors pGM16 and pGM17. The promoterless phleomycin/bleomycin resistance gene (*bleI*) originating from plasmid pUT58 [11] was fused as a *Bgl*III fragment with the *Bgl*III/*Bam*HI fragment of pGM9 [10] to give pGM16. The thiostrepton resistance gene (*tsr*) of pGM9 was replaced by a fragment carrying the chloramphenicol resistance gene (*cat*) from plasmid pJ877 [12] resulting in pGM17.

Abbreviations: rep, replication region of pSG5; aphII, neomycin resistance gene of Tn.5; bleII, bleomycin resistance gene of Tn.5;

P-aphII, neomycin resistance gene promoter

Plasmid fragments including this minimal replicon were combined with various marker genes to construct the pGM vectors [10]. Whereas the *Streptomyces* strains of academic interest, *S. coelicolor* A3(2) and *S. lividans*, are sensitive against the normally used antibiotics neomycin and thiostrepton, some industrially important strains are resistant against these antibiotics. We therefore included – beside others – resistance genes for chloramphenicol [12] and phleomycin [11] in vector construction to extend the applicability of the pGM vectors (Fig. 1).

The most important characteristic of all pGM vectors is their ts-replication which was not achieved by mutagenesis but was found to be an endogenous property of the pSG5 replicon. The pGM vectors are stably inherited at growth temperatures below 34 °C, but they can be efficiently eliminated from their host cell by incubation above 36 °C [10, 13]. Sequence analysis of the 1.6 kb minimal replicon revealed only one single open reading frame (ORF). The molecular mass of the deduced polypeptide was calculated to be 41.8 kD. The amino acid sequence of the protein displayed no significant similarity to any published sequence, not to gene products of the *S. lividans* plasmid pIJ101 [14] neither to any other protein known to be involved in plasmid replication. The protein contains, however, a consensus sequence typical for Rep-proteins of plasmids of Gram-positive bacteria which replicate via single-stranded (ss) intermediate [15]. We therefore concluded that the ORF codes for a Rep-protein involved in pSG5 replication.

As expected from the sequence data, *Streptomyces* harbouring pGM vectors accumulated ss DNA. Since no ss DNA could be detected in *S. ghanaensis* carrying pSG5, it can be assumed that the minus-origin required for lagging strand synthesis has been deleted during vector construction [16].

In addition to its broad host-range and its ts-replication, pSG5 possesses another peculiarity: its minimal replicon is self-transmissible with a transfer frequency of about 10^{-7} . Thus it is likely that the Rep-protein is also involved in plasmid transfer. By comparing the transfer frequencies of different pSG5-derivatives, a DNA fragment of about 600 bp adjacent to the replication region could be identified which enhances the transfer frequency by a factor of 1000.

1.2 The pGM plasmids are suitable vectors for reverse cloning

The properties of the Rep-protein make the pGM vectors suitable cloning vectors for both, conventional cloning and reverse cloning. Using pGM vectors a variety of *Streptomyces* genes has been cloned, such as biosynthetic and resistance genes for phosphinothricin-tripeptide (PTT) and glutamine synthetase (GS) genes [17–19].

However, the major advantage of these vectors arises from the possibility to eliminate autonomously replicating plasmids after exerting a temperature shift. Cells selected for the presence of the vector marker at non-permissive temperatures carry the plasmid integrated in the chromosome. Since pGM vectors themselves are not able to integrate (or only with extremely low frequencies), solely plasmids with a cloned fragment, displaying a region of homology, can integrate into the chromosome. If the cloned piece of DNA consists of an internal part of a transcription unit, integration leads to the disruption of a gene or an operon. Thus, pGM vectors can be used to investigate the function of a cloned DNA fragment by the construction of certain

mutants. The pGM vectors can also be employed for gene replacement and gene rescuing [10].

I.3 *Non-replicative plasmids can be transferred into Streptomyces and subsequently integrated either by transformation of single-stranded DNA or by conjugation*

In Gram-negative bacteria non-replicative plasmids can be used for suicide and integration techniques [20]. Several attempts to transfer these methods to *Streptomyces* led to rather low efficiencies, probably due to the restriction barrier.

But we could show that employment of ss instead of double stranded (ds) DNA may help to solve this problem. *Escherichia coli* vectors with *Streptomyces* marker genes were constructed which contain the replication origin of phage f1. They can therefore be isolated in the ss DNA form from *E. coli* with the use of a helper phage [21]. The transformation frequencies of ss and ds DNA are in the same order of magnitude, but ss DNA is integrated much more efficiently than ds DNA [22] if regions of homology were provided by cloning of *Streptomyces* DNA.

From conjugation of shuttle plasmids from *E. coli* to *Streptomyces* it is known that transfer between these organisms is possible [23, 24]. After cloning the *oriT* of the broad-host range plasmid RP4 [25] into an *E. coli* vector, the resulting plasmids can be mobilized to *Streptomyces* using a suitable *E. coli* donor, such as S17-1 [26] which carries the integrated RP4 plasmid.

E. coli vectors carrying the *oriT*, a *Streptomyces* marker and an internal part of an operon can be conjugated from *E. coli* to *Streptomyces* and selected after integration into the corresponding operon by the presence of the marker. As in the case of the pGM and the non-replicative ss vectors, integration of the plasmid via homologous recombination generates a physically marked mutation.

II. *Establishment of transferred DNA in Streptomyces*

All three systems (transformation of ts *Streptomyces* plasmids, transformation of non-replicative ss *E. coli* vectors, or conjugation of mobilizable non-replicative plasmids from *E. coli* to *Streptomyces*) can be employed for the investigation of *Streptomyces* genes by reverse cloning. The constructed disruption (or replacement) mutants can directly be subjected to genetic and biochemical analyses. Furthermore, application of this technique yields information on the mechanisms involved in transfer and establishment of DNA in *Streptomyces*.

Experiments with different *Streptomyces* strains (*S. ghanaensis*, *S. viridochromogenes*, *S. lividans*, *S. coelicolor* "Müller" and *S. hygroscopicus*) and different genes (three protease genes, PTT-biosynthetic and resistance genes, glutamine synthetase genes and a lysozyme gene) delivered a rather uniform picture of the integration process in *Streptomyces*.

II.1 *Transferred DNA is integrated with high efficiency, often in multiple copies*

The observed recombination frequencies were very high. Using the pGM vectors up to 80% of the cells carried – after a temperature shift – an integrated plasmid. At permissive temperature the plasmids can easily desintegrate. Therefore gene disruption mutants, which were constructed with pGM vectors are rather unstable

without selection pressure (temperature, antibiotic). This problem did not exist for non-replicative plasmids where marker selection guarantees a stable mutant phenotype.

Surprisingly, in many cases integration of the plasmids into the chromosome results in multiple copies. Using the same plasmid, mutants which differ in the number of integrated plasmids were obtained. At present, we do not know the reason(s) for these multiple integrations.

They may originate from

- amplification following the integration,
- integration of plasmids in multimeric form,
- repeating integration processes.

II.2 High gene replacement rates can be obtained using temperature-sensitive vectors

The instability of the disruption mutants which were constructed with pGM vectors can be overcome by generation of replacement mutants. To replace the wild type allele by a mutant allele two recombination events are necessary, which lead to plasmid loss. In other bacteria, the discovery of this rare event requires convenient markers, of which the loss can positively be selected.

To investigate whether such markers are also necessary in *Streptomyces*, we determined the ratio between disruption (one crossover) and replacement (two crossovers) events.

As a test system, a thiostrepton resistance (*tsr*) cassette was inserted into a fragment of the peptide synthetase A gene *phsA*, a PTT-biosynthetic gene of *S. viridochromogenes* [19]. The resulting *phsA*::*tsr* fragment was inserted into a pGM-vector (Fig. 2). Following transformation into *S. viridochromogenes* and selection for thiostrepton resistance at non-permissive temperature, the integration mutants were investigated with regard to single and double crossover. A surprisingly high number of the tested mutants (>50%, depending on the cultivation time) were replacement mutants generated by double crossover. Prolonged incubation of disruption mutants (generated by single crossover) gave rise to replacement mutants by a second crossover event.

These results could be confirmed by analysing the lysozyme gene of *S. coelicolor* "Müller" [27] in a similar way.

However, completely different results were obtained, if similar experiments were performed with single-stranded non-replicative plasmids. Mainly disruption and only a few replacement mutants (~3%) could be found. Furthermore, it was not possible to isolate replacement mutants (second crossover) by prolonged incubation of disruption mutants.

From this we conclude, that there is a strong selection pressure for a second crossover and plasmid loss, if the *ts* *Streptomyces* plasmids are integrated into the *Streptomyces* chromosome. This may be due to the interaction of chromosomal and plasmid replication. Therefore the *ts* plasmids can directly be used for replacement experiments without need for a marker, of which loss can positively be selected.

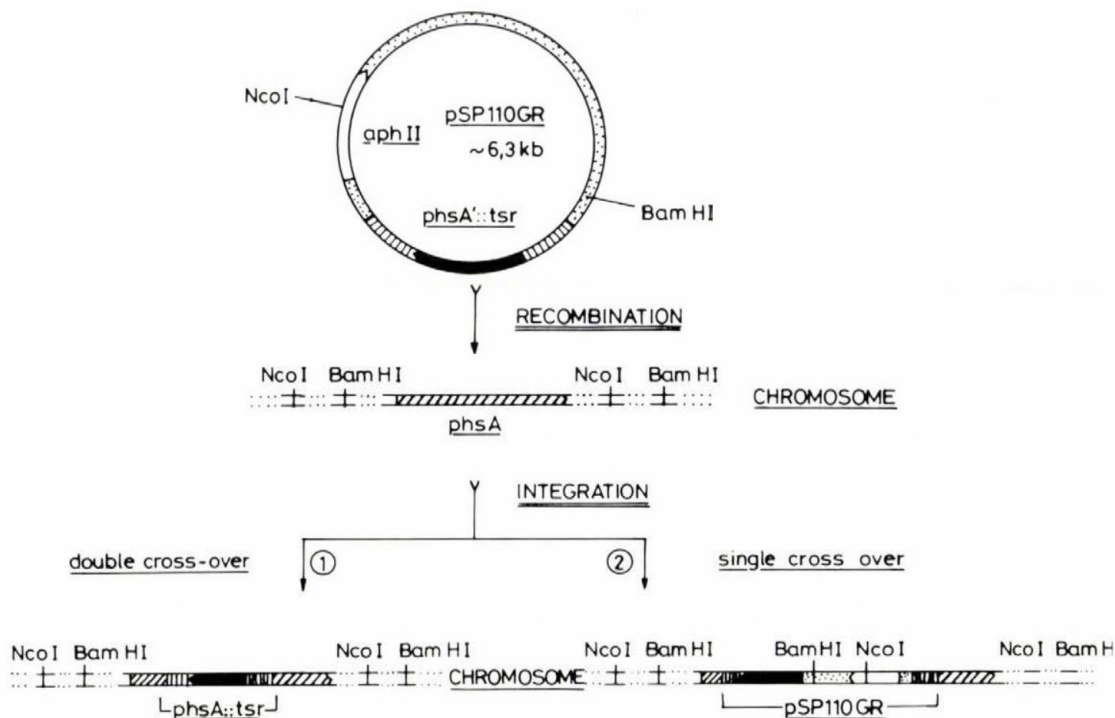


Fig. 2. Scheme of integration of plasmid pSP110GR leading to gene disruption or gene replacement. Vector pSP110GR, which consists of the ts vector pGM11 and an internal *phsA* fragment (*phsA'*) harbouring a *tsr*-cassette, can integrate via single (2) or double (1) crossover into the *S. viridochromogenes* chromosome. The resulting mutants are gene disruption (2) or gene replacement (1) mutants, respectively

II.3 Transferred DNA can integrate "semi-specifically"

Unspecific integration via plasmid sequences has never been observed with the above-mentioned plasmids. However, the regions of homology required for recombination can be very short. Fragments of 200 bp (probably even less) are sufficient to get integration via recombination. Even fragments which do not possess complete homology could be integrated in the following cases:

- The *S. hygroscopicus* *bar* gene [28] can be disrupted by the *S. viridochromogenes* *pat* gene [17] which has an 87% DNA-identity.
- An 0.5 kb fragment of a serine protease B gene of *S. coelicolor* "Müller" could be used to construct two different protease-deficient *S. coelicolor* "Müller" mutants, by disruption of the serine protease A and the homologous serine protease B genes, respectively [29].
- In *S. viridochromogenes* an internal fragment of the *phsA* gene could integrate into another gene of the PTT-biosynthesis which is not located in the known cluster.

These experiments show that the correct integration has to be verified by hybridization in any case, since integration in similar genes (sometimes leading to a similar phenotype) can occur.

On the other hand, this "semi-specific" integration allows the identification of homologous genes and can be a powerful tool for the mutation and isolation of such genes.

III. Recombination

From all these results it can be concluded that most *Streptomyces* strains have an efficient recombination system.

Therefore, we started to isolate the *recA* gene from *Streptomyces*. After comparing all published RecA-sequences, four regions could be identified where the amino acid sequences were highly conserved. Taking the biased codon usage of *Streptomyces* into consideration, four oligonucleotides were designed. Using the PCR technique a 466bp-fragment of *S. cattleya* could be amplified.

It could be proved by hybridization that the amplified sequence originated from *S. cattleya* and that homologues were present in all tested *Streptomyces* strains. DNA-sequence analysis revealed the typical structure of a coding DNA-fragment of *Streptomyces* with an overall G+C-content of 66.5%. Comparison with EMBL gene bank showed significant similarity (60–80%) to various *recA* genes from Gram-positive and Gram-negative bacteria. The deduced amino acid sequence displayed the highest similarity (85.3%) to the *Mycobacterium tuberculosis* [30] RecA gene product.

The 466bp-fragment will now be used to construct disruption mutants and to isolate the complete *recA* gene from *Streptomyces*.

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FUNCTIONS OF SYMBIOTIC FUNGUS GARDENS IN HIGHER TERMITES OF THE GENUS *MACROTERMES*: EVIDENCE AGAINST THE ACQUIRED ENZYME HYPOTHESIS

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Behavioural, microbiological and biochemical studies on *Macrotermes subhyalinus* and *M. michaelseni* by collaborating laboratories in the U.K., Switzerland and Australia are described. Younger workers consume both primary forage and the conidia of a symbiotically associated fungus of the genus *Termitomyces*, but all workers produce a fully competent cellulase complex (endoglucanase + glucosidase) in the midgut which is clearly distinguishable from analogous enzymes in fungal tissues. Workers have a RQ of 1.0; although a bacterial flora is present, assessments of CH₄/H₂ efflux and intestinal VFAs suggest that respiration is sustained by aerobic carbohydrate dissimilation. Calculations based on estimates of food ingestion by workers and measurements of cellulase activity show that endogenous production of reducing sugars from polysaccharide is sufficient to sustain the observed metabolic rate. Conidia contain both cellulase and glucose at much higher concentrations than other fungal tissues, but the role and fate of these substances on entering the young worker guts is unknown. Older workers consume fully composted forage in which cellulose, hemicellulose, pectin and lignin are all significantly degraded, with a corresponding increase in nitrogen content.

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The traditional view that termites must form obligate associations with micro-organisms has come under critical scrutiny following recent demonstrations that endogenous cellulases are produced in many species [1-4]. Nevertheless, all termites appear to lose viability after separation from some or all of their microbial partners [5-7] and the extended hindgut, containing many protozoa and/or bacteria, is present in the worker castes of all forms [8].

The subfamily *Macrotermitinae* is one of the most interesting of the higher termites partly because they cultivate fungus gardens of the genus *Termitomyces* but also because of the greater division of labour amongst the workers [9].

Suggestions for the role of the fungus include (i) that it degrades lignin in forage gathered by the termite host [10] (ii) that composting of forage concentrates nitrogen and improves termite nutrition [11] and (iii) that the termite can ingest fungal cellulolytic enzymes located in conidia to complement its own digestive enzymes [12]. In support of (iii) Rouland et al. [4], working on *Macrotermes mulleri* suggested a synergistic interaction between cellulolytic enzymes of termite and fungal origin in the gut, but the data of Veivers et al. [13], derived from *M. subhyalinus* and *M. michaelseni*, show that 90% of the glucose requirements of workers can theoretically be met by the action of endogenous intestinal cellulases. It is unclear whether these relationships reflect the absence of a symbiotic association with intestinal bacteria.

Although the literature contains many investigations of symbiotic associations based on the experimental separation of the partners and subsequent examinations of their biochemical capabilities *in vitro*, this approach has been unproductive in higher termites. One reason may be that the bacterial populations of termite guts, like many other natural assemblages of micro-organisms in favourable but physically confined habitats, are consortial in nature; hence their functions, if any, are best resolved by an examination of the system as a whole. In this paper the overall intestinal mechanisms of worker castes in two common African species of *Macrotermes* are characterized to assess the possible role of gut bacteria and clarify the contribution of ingested fungal enzymes in the digestion of cellulosic material.

Termite caste differentiation and behaviour

On the basis of morphology, foraging and feeding habits, four categories of workers can be distinguished in *M. subhyalinus* [14] and *M. michaelseni* [15] the foragers are old majors and minors (more than 30 days old), with the young major and minor workers (less than 30 days) involved in processing the forage, construction of the fungal comb and feeding of dependent castes (Fig.1). Young workers consume both primary forage and ripe fungal conidia, but the food retention time is short and the contents of the gut, which appear to be lightly digested, are rapidly incorporated into the fungus comb [16]. Old workers consume senescent comb after sporulation has subsided, together with some mineral soil and plant material. Resolution of the symbiotic system therefore depends on a full recognition of this social differentiation in experimental work.

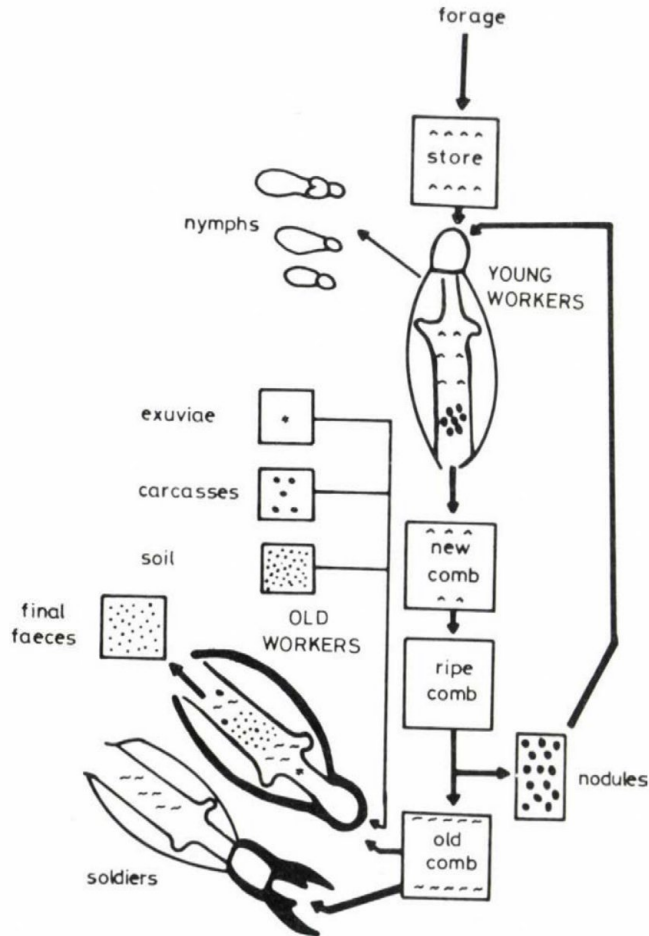


Fig. 1. Simplified scheme showing the processing of forage by termites of the genus *Macrotermes*, and the sequence of feeding by young and old workers. Old workers collect the forage from the field and consume old fungus comb. Young workers consume both newly collected forage and ripe fungal conidia (nodules). Both young and old workers occur in major and minor morphological castes. Storage of forage prior to consumption by young workers varies amongst the species of this genus

Gut morphology and physiology

The general arrangement of the alimentary canal is similar in all four worker castes (Fig. 2), but the total volume is greater in majors (averaging $6.81 \pm 2.20 \mu\text{l}$; $n=20$) compared with $3.46 \pm 1.23 \mu\text{l}$ in minors. The component sections make up differing proportions of the total in majors and minors. Thus in major workers the midgut and hindgut make up 64.6% and 29.4% of the total volume respectively, while in minors the equivalent contributions are 53.0% and 40.5%. In relative terms, therefore, the

midgut is a more prominent structure in major workers and differentiation of these castes involves an allometry in the alimentary canal.

Intestinal pH (determined with narrow range indicator paper) is within one unit of neutrality in the crop, midgut, colon and rectum, with no evidence of variation between castes, but the paunch region is consistently alkaline with pH estimates up to 9.5 in young major and young minor workers. Determinations of redox potential in young worker guts (estimated from the colour changes of ingested indicator dyes in an observation colony) indicated aerobic conditions in the crop and midgut, but in the paunch, colon and rectum both thionine and methylene blue were reduced, indicating mildly reducing microaerobic conditions. Nile blue ($E'_{\circ}$ at pH 7, -123 mV) retained its oxidized colour throughout the hindgut and allowing for the dependence of $E'_{\circ}$ on pH (dyes are more readily reduced under alkaline conditions), mean hindgut redox potential is unlikely to be more negative than -123 mV.

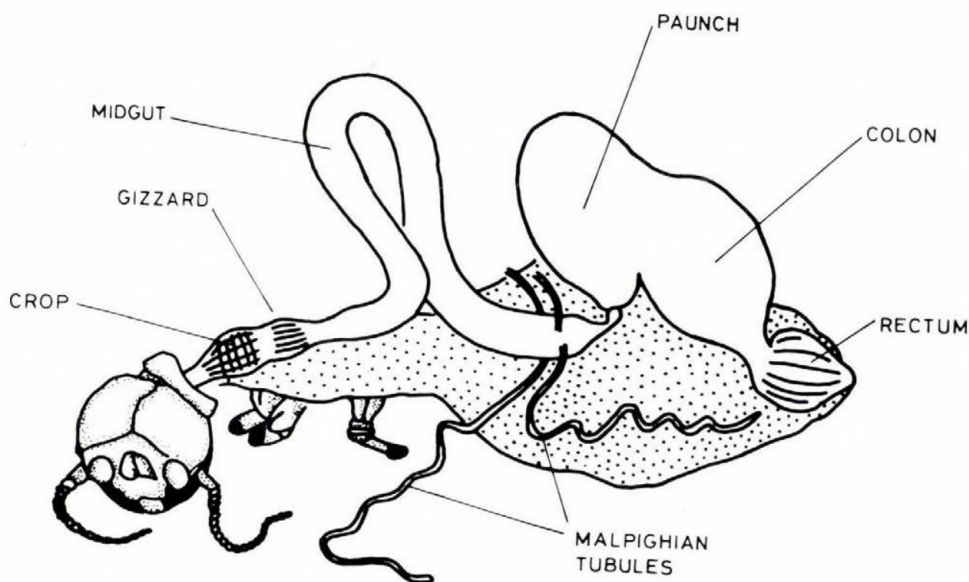


Fig. 2. Morphology of the gut in worker castes of *M. subhyalinus* and *M. michaelseni*

Gas exchanges

RQ values (determined by the Warburg method) for young and old major workers of *M. michaelseni* were 0.999 ± 0.007 (SD, $n=4$) and 1.002 ± 0.005 (SD, $n=5$) respectively. Oxygen consumption by workers (determined by gas chromatography from samples of 100 termites) averaged 7.175 nmol/mg/h, ranging from 5.89 ± 3.21 nmol/mg/h (SD, $n=6$) in young majors to 7.81 ± 1.67 nmol/mg/h in old majors.

Table I

Rates of efflux of carbon dioxide, hydrogen and methane from living worker castes of M. subhyalinus

Caste	Gas efflux						Molar ratio
	CO ₂		H ₂		CH ₄		
	volume	moles	volume	moles	volume	moles	
Young minors	0.22 ±0.06	9.82 ±2.49	0.0010 ±0.0002	0.044 ±0.007	0.0004 ±0.0001	0.016 ±0.004	0.16
Young majors	0.18 ±0.04	8.04 ±1.87	0.0005 ±0.0001	0.024 ±0.006	0.0003 ±0.0001	0.016 ±0.003	0.20
Old minors	0.21 ±0.07	9.38 ±3.05	0.0019 ±0.0006	0.081 ±0.029	0.0031 ±0.0008	0.141 ±0.037	1.51
Old majors	0.18 ±0.10	8.04 ±4.45	0.0028 ±0.0005	0.125 ±0.025	0.0018 ±0.0005	0.079 ±0.021	0.98

To permit ready comparisons, outputs are given in both volumetric ($\mu\text{l/h/mg}$) and molar (nmol/h/mg) units. Mean $\pm$ S.D. of gas efflux ($n = 6-8$). Emissions were determined by gas chromatography from samples of the atmosphere above 100 workers of each caste contained in a tared, sealed 25 ml Erlenmeyer flask at 25 °C. CO₂ was determined in a Pye Unicam PU 4900 GC fitted with a Poropak-T column with helium as carrier gas at 30 ml/min. CH₄ and H₂ were determined in a Perkin Elmer Sigma 3B dual column GC. A Poropak-T column retained CH₄, which was then diverted to a flame ionization detector; H₂, with other atmospheric gases excepting CO₂, was passed to a Molecular Sieve 5A column and after separation was detected by thermal conductivity. Argon was used as carrier at 30 ml/min and 100 °C.

Live specimens of the worker castes emit detectable quantities of hydrogen and methane, in addition to carbon dioxide (Table I). Mean hydrogen efflux ranged from 0.024 nmol/mg/h in young majors to 0.125 nmol/mg/h in old majors, with intermediate rates in the minor castes. Methane efflux was lowest in young major and young minor workers, averaging 0.016 nmol/mg/h in each case, but increased in older workers to 0.079 nmol/mg/h in the major and 0.141 nmol/mg/h in the minor caste. The rates for older castes are close to the lower limits of methane emission reported for lower termites (e.g. Fraser et al. [17]). There are few reports of hydrogen efflux in the modern literature, but Odelson and Breznak [19] estimated production in *Reticulitermes flavipes* as 0.003 to 0.006 $\mu\text{l/mg/h}$, a similar figure to that obtained in the present study for old minor workers. Table I also shows that carbon emitted as methane by *M. subhyalinus* is a very small proportion of the carbon respired as CO₂, the highest molar ratio determined for the two gases was a mere 1.51% in old minors. At present we have no information on methane production, if any, by the fungus garden.

Microbiology and end products

The total densities of prokaryotic intestinal organisms are set out in Table II. This summarizes a detailed microscopical study of the gut floras of worker caste termites in *M. subhyalinus* and shows that the greatest densities, of approximately 1.3×10^{11} organisms/ml occur in the paunch and colon of old minors, with lower densities in other castes, particularly the young majors. These densities are broadly in line with the limited data available for other higher termites (e.g. Bignell et al. [18]), but reveal little about the functional role of gut bacteria. The intestinal flora is heterogeneous, with rod, coccoid and spirochaete morphotypes recognizable in most guts. Filamentous prokaryotic micro organisms were not observed.

Table II

Mean total densities of bacteria (organisms per ml) in the guts of four worker castes of M. subhyalinus

Gut section	Caste	Bacterial density, organisms ml ⁻¹
Midgut	Young minor	6.5×10^9
	Young major	2.8×10^9
	Old minor	3.0×10^9
	Old major	2.3×10^9
Paunch	Young minor	1.0×10^{10}
	Young major	5.2×10^9
	Old minor	1.3×10^{11}
	Old major	5.9×10^{10}
Colon	Young minor	6.9×10^{10}
	Young major	3.1×10^{10}
	Old minor	1.3×10^{11}
	Old major	5.8×10^{10}
Rectum	Young minor	2.3×10^{10}
	Young major	8.5×10^9
	Old minor	6.8×10^{10}
	Old major	3.7×10^{10}

Total density determined by direct observation using the agar film method (Bignell et al., [18]). Gut sections were separately pooled from 6 worker termites of each caste, homogenized in 1 ml of sterile 0.1% peptone water and mixed with an equal volume of melted ion agar at 45 °C prior to preparation of the films.

Of the volatile fatty acids expected to be formed by the anaerobic dissimulation of cellulosic materials (acetate, butyrate, propionate), only acetate was detected in gut homogenates (Table III). However the concentrations were low, the highest observed being 0.46 mM in old minors; this is more than two orders of magnitude less than the

concentrations reported for hindgut fluid in lower termites (e.g. Odelson and Breznak [19]), a difference too great to be accounted for by the dilution effect of including the midgut in the homogenates. Consequently there is little evidence from VFA accumulation that significant anaerobic degradation of cellulosic materials occurs, although when the acetate content of gut homogenates is normalized to fresh body weight the acetate pool more than doubles in size in both major and minor workers from the young to the old age categories. Few data are available on VFA concentration in higher termite guts, except in *Nasutitermes walkeri*, where acetate is reported as 8 mM (Williams et al. [20]). This termite appears to utilize bacterially generated acetate to support its respiration, but is not a species associated with fungi. Small amounts of pyruvate and lactate were detected in the gut homogenates of *M. subhyalinus* (Table III).

Table III

Concentrations of acetate, pyruvate and lactate in gut homogenates of four worker castes of *M. subhyalinus*

Caste	Concentration, mM			Acetate normalized as nmol/mg body weight
	Acetate	Lactate	Pyruvate	
Young minor	0.11±0.05	n.d.	< 0.04	0.59
Young major	0.18±0.02	< 0.11	< 0.04	0.91
Old minor	0.46±0.05	n.d.	< 0.04	1.83
Old major	0.37±0.04	< 0.07	< 0.04	2.01

n.d. – not detected.

Fifteen guts were pooled for each determination. Mean ± S.D. of 3 determinations. Acids were determined by HPLC at room temperature using a LKB Broma-Gerates chromatograph fitted with a HPICE-AS1 column. Following homogenization of the guts in 1 ml 0.1 M phosphate buffer, pH 7.0, and centrifugation at 3000 g for 10 min, homogenates were filtered through a 0.25 µm membrane and eluted through the column with 1 mM HCl or 1 mM HCl containing 5 mM TDFHA at a back pressure of 43–58 bar and flow rate of 0.6–0.8 ml/min. UV detection was employed for all acids.

Cellulases

Tables IV–VI summarize work on the cellulase enzyme complexes of both termite hosts and fungal associates, which is more fully reported elsewhere (Veivers et al. [13]). Table IV shows that substantial activity from cellulase and its components was found in all four worker categories of *M. michaelsoni*. There was no significant difference (90% level of confidence) in cellulase activity between the four castes, whether this is expressed in units/termite/h, as in Table IV, or as units/mg termite/h (fresh weight), excepting young majors whose activity is significantly greater on the basis of units/mg termite/h. In *M. subhyalinus* (not shown in the tables) young majors and young minors have more activity than the old categories. Larvae and soldiers have little or no cellulase activity in either species (also not shown).

At least 99% of the cellulase activity was found only in the midgut of both young and old workers, although about 8% of the endo- β -1,4-glucanase activity occurs in the salivary glands (Table V). The only fungal material associated with the termites with substantial enzyme activity is the conidial nodules.

Table IV

Enzyme activities in worker castes of M. michaelseni, and in associated fungal material (n = 3)

Source	Cellulase (units)	Endo- β -1,4-glucanase (units)	β -1,4-glucosidase (units)
Old major	21.9 $\pm$ 4.8	11.7 $\pm$ 1.8	4.55 $\pm$ 0.16
Young major	28.1 $\pm$ 2.2	23.4 $\pm$ 2.0	8.23 $\pm$ 2.14
Old minor	18.2 $\pm$ 3.4	10.3 $\pm$ 3.6	4.26 $\pm$ 0.41
Young minor	19.5 $\pm$ 3.1	12.0 $\pm$ 7.9	4.74 $\pm$ 1.39
Fungal comb	0.03 $\pm$ 0.01	0.02 $\pm$ 0.01	< 0.01
Nodules	8.22 $\pm$ 0.86	3.57 $\pm$ 1.33	< 0.01

Termite homogenates were prepared from pooled samples of 10 whole insects in 1 ml of 0.1 M acetate buffer, pH 5.5, at 4 °C. Fungal material (100–200 mg) was also homogenized in 1 ml buffer and sonicated for 30 s at 40W; nodules (10–20 mg) were similarly treated in 0.5 ml buffer. Supernatants were collected from homogenates centrifuged for 10 min at 9800 g and assayed for enzyme activity. β -1,4-glucosidase and endo- β -1,4-glucanase were assayed by the method of Hogan et al. [21]; cellulase was assayed by incubating 0.2 ml of 20% Sigmacell suspension with 1 ml extract for 1 h at 37 °C. Glucose produced was estimated by glucose GOD-Perid reagent.

Cellulase assay: a unit is defined as activity which produced 1 μ g glucose/termite/h $\pm$ SD or 1 μ g glucose/mg wet weight fungal tissue/h $\pm$ SD from Sigmacell

Endo- β -1,4-glucanase assay: a unit is defined as activity which produced 1 mg reducing sugars (glucose equivalents)/termite/h $\pm$ SD or 1 mg reducing sugars (glucose equivalents)/mg wet weight fungal tissue/h $\pm$ SD from carboxymethylcellulose (CMC)

β -1,4-glucosidase assay: a unit was defined as activity which produced 1 mg glucose/termite/h $\pm$ SD or 1 mg glucose/mg wet weight fungal tissue/h $\pm$ SD from cellobiose.

When elution profiles (by column chromatography on Bio-Gel P-60) of the endo- β -1,4-glucosidase activities from the fungal nodules and from old and young major workers of *M. michaelseni* were compared, there was little overlap between the fungal and the termite enzymes. A similar result was obtained for β -1,4-glucosidase (using Bio-Gel A-0.5 m); there are at least two β -1,4-glucosidase activities in the fungal nodules which are distinct from the two in the major workers of *M. michaelseni*. Elution profiles of endo- β -1,4-glucanase activities from the major workers also indicate a multi-enzyme system.

Fungal nodules were found to contain a large amount of glucose (Table VI). The significance of this finding is unknown, but it was shown that fungal β -1,4-glucosidase was not inhibited in vitro by glucose concentrations up to 1 mM.

Table V

Distribution of enzyme activity in gut sections of young and old major workers of *M. michaelseni*, $n = 2$

Source	Cellulase %	Endo- β -1,4-glucanase %	β -1,4-glucosidase %
<i>Old majors</i>			
Salivary glands	1.2 $\pm$ 0.1	7.2 $\pm$ 1.2	0.4 $\pm$ 0.0
Foregut	< 0.1	0.7 $\pm$ 0.3	0.2 $\pm$ 0.3
Midgut	98.8 $\pm$ 0.1	89.7 $\pm$ 2.1	98.2 $\pm$ 0.9
Hindgut	< 0.1	2.4 $\pm$ 0.7	1.2 $\pm$ 0.6
<i>Young majors</i>			
Salivary glands	< 0.1	9.0 $\pm$ 0.3	0.5 $\pm$ 0.6
Foregut	< 0.1	0.4 $\pm$ 0.1	0.5 $\pm$ 0.6
Midgut	99.7 $\pm$ 0.5	85.8 $\pm$ 2.5	93.6 $\pm$ 4.1
Hindgut	0.3 $\pm$ 0.5	4.8 $\pm$ 2.2	5.4 $\pm$ 2.8

For each enzyme the activity is shown as a % of the total activity in the alimentary canal as a whole. The enzymes determined are defined as in Table IV.

Table VI

Sugars in freeze-dried extracts of gut sections of *M. michaelseni* and associated fungal material, as nmol/termite or nmol/mg fungal dry wt

Source	Glucose	Cellobiose	Xylose	Arabinose
<i>Old majors</i>				
Midgut	47 $\pm$ 11	5 $\pm$ 8	0	0
Hindgut	15 $\pm$ 5	trace	0	0
<i>Young majors</i>				
Midgut	37 $\pm$ 9	8 $\pm$ 4	trace	0
Hindgut	4 $\pm$ 1	2 $\pm$ 2	0	0
Fungus comb	13 $\pm$ 3	5 $\pm$ 3	15 $\pm$ 8	24 $\pm$ 3
Nodules	184 $\pm$ 83	trace	0	0

Sugars were determined by thin layer chromatography on aluminium-backed silica gel plates after homogenization (guts) or sonication (fungus) in distilled water, deproteinization with perchloric acid and neutralization with KOH [13]. Lyophilized supernatants were redissolved in distilled water, desalted with Amberlite MB-3 and treated with polyamide to remove phenolic compounds.

If the termites need to ingest a cellobiohydrolase to facilitate cellulose digestion, as suggested by Martin [12] for *M. natalensis*, or an endo- β -1,4-glucanase as implied by Rouland et al. [4] for *M. mulleri*, then it is to be expected that only fungal material containing the activity would be ingested. This is not the case for *M. subhyalinus* and *M. michaelseni* as our results indicate that 99% of the fungal cellulase activity is found in the nodules, yet these are eaten only by the young major and young minor workers. Even in these castes the estimated rate of consumption of nodules (9 $\mu\text{g/h}$ in *M. subhyalinus*, based on unpublished observations) would be sufficient to deliver only 0.03% of the total cellulase activity found in the termites. The rate of consumption of the (old) comb material by *M. michaelseni* is 0.015 mg (dry wt)/mg termite (wet wt)/day (after Darlington [15]). Thus the amount of glucose consumed by old workers eating senescent comb would be 0.15 nmol/termite/h, an insignificant amount in comparison with the amount of glucose which can be produced by the termite's cellulase (0.12 $\mu\text{mol/termite/h}$).

Discussion

On the assumption that glucose is oxidized aerobically to sustain respiration, the rate of production by the digestive system to support an O_2 consumption of 7.175 nmol/mg/h at 25 °C would be 1.196 nmol/mg/h, or approximately 14 $\mu\text{g/h}$ for a major worker weighing 12 mg and 10 $\mu\text{g/h}$ for a minor weighing 8 mg. The rates at which glucose is produced in situ in the midgut are not known, but the activity of endogenous cellulases in midgut homogenates would be sufficient to produce approximately 35 μg of glucose/h in major workers and 19 $\mu\text{g/h}$ in minor workers. On this evidence, therefore, there is no need to propose the involvement of either fungal or bacterial partners in the dissimilatory process. The assumption of aerobic carbohydrate-based respiration is supported by the observed RQ of 1.0, the low levels of methane and hydrogen efflux, and by the relatively small accumulation of acetate in gut fluids. An RQ of approximately 1.0 could be obtained if cellulose were fermented to acetate, CO_2 and H_2 by a component of the gut flora, while another component carried out anaerobic acetogenesis from CO_2 and H_2 and the termite host aerobically oxidised all the acetate produced from both sources. However the low concentrations of acetate accumulated and the absence of strongly reducing conditions required for prokaryotic acetogenesis argue against this possibility. Such CH_4 and H_2 as is evolved by the termites can be attributed to the formation of anaerobic microsites within the hindgut, a normal phenomenon in many animals which does not necessarily imply a symbiotic mechanism.

The role of the fungus gardens in *Macrotermitinae* remains unknown. Although the investigation of cellulose digestion has stimulated elegant research and brought to light many interesting features of the biology of the termites, it has so far failed to reveal the true nature of the relationship between the insects and their fungal partners.

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CHARACTERIZATION OF TURBOT (*SCOPHTHALMUS MAXIMUS*) ASSOCIATED BACTERIA WITH INHIBITORY EFFECTS AGAINST THE FISH PATHOGEN *VIBRIO ANGUILLARUM**

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More than 400 isolates from the intestine and the external surface of farmed turbot, as well as from fish food and hatchery water were screened for inhibitory effects against the fish pathogen *Vibrio anguillarum* HI 11345 and seven other fish pathogens. The bacteria with inhibitory effects were then characterized with regards to their sites of colonization, especially the intestinal regions and sites within each region. No correlations between the different biochemical phena, site of colonization and inhibitory effect could be found. The potential of seven of these strains for adhesion to intestinal mucus from turbot was studied. Rapid growth of *V. anguillarum* in intestinal mucus was measured, hence it is feasible that the intestinal tract is a site for *V. anguillarum* multiplication. Strains isolated from the intestine showed greater capacity for in vitro adhesion to and growth in fish intestinal mucus than did the pathogen and skin mucus isolates. Two of the strains isolated from the intestine were studied for their inhibitory kinetics and one strain for the potential of in vivo colonization. The molecular weight of the inhibitory component was below 1000 dalton in 65% of the strains isolated. For the other 35% the inhibitory component ranged between 1000 D-6000 D in dialysis cut off experiments.

One week after oral administration, one such isolate still accounted for 45% of the total c.f.u. in the intestinal mucus of 5 g turbot. In preliminary experiments we demonstrated that it is possible to detect the pathogen in intestinal mucus with a fluorescently labelled oligonucleotide probe directed against a specific part of the intracellular rRNA of *V. anguillarum*.

A major problem in fish aquaculture is the invasion of bacteria that are pathogenic to the fish. The loss of juvenile turbot in Norwegian fish hatcheries as a result of vibriosis was 40% in 1988 [1]. Although some protection is obtained by the use of antibiotics, chemotherapy and vaccination, more efficient means are needed for controlling bacterial pathogens.

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The use of antibiotics for therapeutic treatment of fish diseases in Norwegian aquaculture has increased along with the increase in fish production. In 1991 more than 35 tons of antibiotics were used, compared to less than 5 tons in 1980 [2]. The environmental impact of using huge amounts of antibiotics is unacceptable. As a result of using drugs such as sulfonamides, chloramphenicol, tetracycline and nitrofurantoin derivatives [3], the natural bacterial population of water and sediment displays an increased multiple antibiotic resistance, presumably due to the rapid transfer of resistance mediated by plasmids [3-4].

An alternative prophylactic and therapeutic treatment could be to support the natural host microbial defence system by administration of live bacteria with demonstrable inhibitory effects against pathogens. This concept, often referred to as probiotics, has already been applied for farming of animals (reviewed by e.g. Fuller [5]).

It has been shown that the disturbance of the host gut microflora induces increased sensitivity to pathogenic infections, an effect which can be reduced by administration of bacteria with inhibitory activity against the pathogen. It has become apparent that it is desirable for such bacteria to be of host origin and to have the capacity to colonize the gastrointestinal tract for protection to occur (reviewed by e.g. Conway [6]).

In several studies, it has been suggested that an indigenous microbial flora exists in the digestive tract of fish. Campbell et al. [7] showed that the distribution of bacteria in the intestine of farmed Dover sole was not consistent with that of the water nor the fish diet microflora. For example, recent studies have shown that the gastrointestinal tract of marine fish of various species contains bacterial strains that belong to two specific *Vibrio* groups. These strains were suggested to inhibit growth of *V. anguillarum* and *V. salmonicida* (Onarheim et al. [8]).

It may be feasible to identify intestinal strains which can be orally administered to turbot and thereby protect the fish from *V. anguillarum* infection. While the point of entry of the pathogen into the fish is still not determined, the gastrointestinal tract has been implicated as a site of amplification and colonization [9]. In Pacific salmon naturally infected with *V. anguillarum* and *V. ordalii*, the pathogens were found in the pyloric caeca and throughout the intestinal tract, which supports the notion of the gastrointestinal tract as an important site in epizootics.

Based on the results of inhibitory effects on bacterial pathogens by constituents of fish gut microflora [8] and by specific marine epiphytic strains isolated from seaweed [11] we were interested to initiate similar studies on farmed marine fish such as turbot (*Scophthalmus maximus*).

Materials and methods

Isolation of bacteria. Six turbot (*S. maximus*), water samples and food pellets were collected at MOWI A/S hatchery in Bergen, Norway. The fish had not been treated with antibiotics or other drugs, even though there had been some outbreaks of vibriosis from which the fish had recovered as juveniles. The fish were five years old and their weight ranged between 1.0-1.5 kg. Water was taken 50 cm below the water surface in sterile bottles and spread on marine agar plates. The food pellets were

homogenized in NSS before plating. One fish at a time was killed and mucus from the external surface was sampled using a rubber scraper and suspended in NSS before plating. The stomach and the intestine were dissected out, rinsed in sterile NSS and cut open and intestinal mucus scraped off with a rubber scraper, suspended in NSS and plated. The media and diluent used were Tryptic soy broth (TSB, Difco Laboratories), Marine broth (MB, Difco Laboratories) and artificial seawater (Nine Salt Solution). Different isolates (n=403) were picked from the original plates according to morphology, pigmentation, variability and number in the different samples (Westerdahl et al. [12]). For screening of inhibitory effects against the fish pathogen *V. anguillarum* HI 11345 a double-layer method, previously described by Dopazo et al. [13] was used.

Biochemical characteristics. A marine biochemical identification system of Hansen and Sørheim [14] was used for characterization of the isolated strains to evaluate possible correlations between location, inhibitory effect and the phenon.

Inhibitory studies. The size of the inhibitory components was estimated as follows. Macrocolonies were produced as described above. Two different dialysis membranes (Spectra/pore) of mw cut off 1000 D and 6000 D were washed in distilled water and cut to fit an agarplate. The membranes were placed on top of the macrocolonies after 24 h of growth, prior to applying the double layer technique described above.

The isolates which inhibited growth of *V. anguillarum* HI 11345 were subsequently analyzed for inhibitory effects against the following fish pathogens: *V. anguillarum* R 73 serotype 01 isolated from turbot, *V. anguillarum* 860908-5 serotype 02 isolated from turbot, *V. anguillarum* 1173/1 serotype 02A isolated from cod, *V. anguillarum* 820723-2/8 serotype 02B isolated from salmon, *V. anguillarum* NCMB 2129, *Aeromonas hydrophila* CCUG 1455 and *A. salmonicida* CCUG 2116.

Of the strains with an inhibitory effect, eight were selected for further studies. Seven of them had an inhibitory compound that did not penetrate the 1000 D membrane. These strains were selected due to either a broad inhibitory spectra against several of the fish pathogens, or a very efficient inhibitory effect against *V. anguillarum* HI 11345.

Inhibitory kinetics. Two strains (4:44, 5:28) displaying inhibitory effect by the double layer method, were studied for differences in inhibitory activity during stationary phase. The effect was measured as changes in the absorbance, using a microtiter spectrophotometer (BioTech, Biokinetics). Bacterial cultures were grown in TSB+2% NaCl, which was continually shaking for 2, 4, 10 and 14 days. Supernatant was taken from each culture, filter sterilized and transferred to a microtiterwell. An equal volume of fresh TSB+2% NaCl was added, before *V. anguillarum* cells were inoculated. As an additional control, supernatant of *V. anguillarum* was used.

Growth studies. The growth rate of some intestinal (4:44, 4:45, 4:46) and external isolates was measured in mucus and TSB by monitoring the optical density at 540 nm. The length of the lag period and the generation time for growth of the isolates were also calculated.

A twenty-four hour culture in TSB-NSS of intestinal strain 4:44 was centrifuged and the supernatant filter sterilized. Filtrate and fresh medium were mixed in equal parts and then inoculated with *V. anguillarum*. Growth rates of the pathogen were determined then by monitoring the optical density at 540 nm and generation times were calculated. Flasks with only fresh medium and with NSS instead of filtrate were used as the controls.

Coculturing. Intestinal isolate 4:44 was used in a coculturing experiment with *V. anguillarum*. *V. anguillarum* and strain 4:44 were grown 18 h, then the same number of cells of each culture was inoculated in TSB+2% NaCl. Growth was measured by c.f.u., and the two strains were detected using oxidase test and colony morphology. As controls, pure cultures of the two strains were grown separately and measured in the same way.

Adhesion. A modification (lower incubation temperature) of the method of Laux et al. [15] used for studying adhesion of enteric bacteria to mice intestinal mucus was employed.

Colonization. A spontaneous mutant strain (streptomycin resistant) of isolate 4:44 was used in an in vivo colonization study, using 4 g turbot in an aquarium with a recirculating water system. The colonization potential of 10^8 cells administered via the water or orally, was measured at day 1 and day 7 after administration by sacrificing fish and taking samples from the intestinal mucus.

Four *V. anguillarum* 16 S RNA specific sequences, selected from the literature, were tried for in situ hybridization. This work was done in cooperation with Dr. R. Amann, Technical University of München, Germany. The methods used are described in Amann et al. [16]. The resulting probe was used for detection of the pathogen in infected fish.

Results and discussion

Bacterial isolates. Twenty-two percent of the isolated strains showed inhibitory effect against *V. anguillarum* HI 11345 [12].

Of the total number of bacteria isolated from the intestine (n=283), 28% were inhibitory against growth of *V. anguillarum* HI 11345, while 11% of the bacterial flora obtained from the outer mucus layer (n=76) had inhibitory effects. Furthermore, only 3% of the bacteria in the water (n=34) proved to be inhibitory against *V. anguillarum* HI 11345 (Fig. 1). No inhibitory strains were found in the food pellets (n=10) [12].

Biochemical characteristics. Of the total number of isolates with an inhibitory effect, 93% were negative, 3% Gram-positive and the remaining 4% were not tested. The inhibitory strains varied in taxonomy, but it was possible to discern different clusters. All isolates were sensitive to the vibriostatic agent O/129, 3% displayed elastase production and 94% showed haemolytic activity. No correlations between the different biochemical phenomena, site of colonization and inhibitory effect could be found [12].

Inhibitory studies. Sixty-five percent of the inhibitory strains were found to produce inhibitory components that were less than 1000 D in size, 35% of the isolates did not show any inhibitory effect when the 1000 D membrane was used, while the inhibitory component of all strains was found to pass through a 6000 D membrane.

Sixty percent of the inhibitory strains showed an effect against all *V. anguillarum* strains examined. Three percent were specifically inhibitory against *V. anguillarum* HI 11345, but not to any other *V. anguillarum* serotype. The percentage of inhibitory strains that showed an effect on each specific *V. anguillarum* serotype varied from 100% to 74% of the total number of inhibitory strains. Ninety percent of 85 strains tested showed effect against *A. salmonicida* and 96% of 47 strains tested showed effect against *A. hydrophila* [12].

Growth studies. All isolates grew in intestinal mucus from turbot. Although the mucus was diluted ten-fold (final concentration 0.5 mg protein/ml), it provided the organisms with sufficient nutrients to show similar growth rates and stationary phase optical densities as in TSB. The gut isolates generally had a shorter lag phase, 1.75 ± 0.43 h, and generation time, 54 ± 8 min, than did the skin isolates, 3.33 ± 0.47 h and 115 ± 5 min. In addition, the gut isolates generally grew faster in intestinal mucus than did *V. anguillarum* [17].

The growth of the pathogen was clearly suppressed by components in the supernatants. This suppression is visualized by reduced generation time compared to the control experiment as seen in Fig. 2 (Olsson et al., unpublished studies). In the coculture experiment growth of *V. anguillarum* was strongly inhibited as seen in Fig. 3 (Olsson et al., unpublished studies).

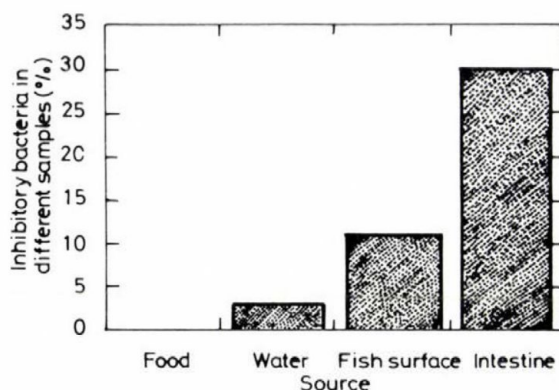


Fig. 1. The percentage of total bacteria with an inhibitory effect against *V. anguillarum* HI 11345 compared with the total number of bacteria isolated from food, water, the fish external surface and the intestine of turbot

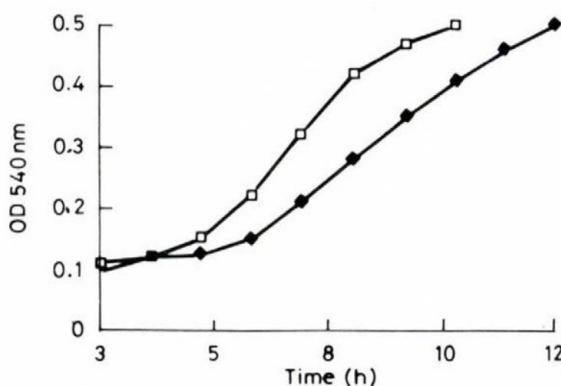


Fig. 2. Growth rate of *V. anguillarum* HI 11345 (—●—) in 50% fresh TSB+2% NaCl+50% supernatant of intestinal isolate 4:44. Growth in 50% fresh TSB+2% NaCl+50% supernatant of *V. anguillarum* (—□—) served as a control

Adhesion. The isolates showed very different adhesion patterns. Turbot isolates 4:44 and 4:46 had two-fold greater adhesion to turbot intestinal mucus than to the control surface BSA, and intestinal isolates adhered much better to all surfaces than did *V. anguillarum* [17].

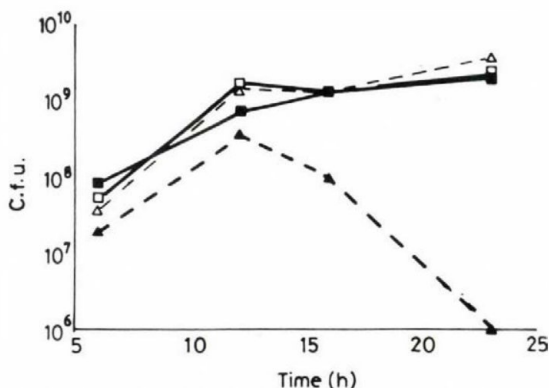


Fig. 3. C.f.u. numbers representing coculture growth in TSB+2% NaCl of *V. anguillarum* (---△---) and intestinal isolate 4:44 (—■—). Separate growth of *V. anguillarum* (---△---) and intestinal isolate 4:44 (—■—) served as controls

Colonization. At day 1, 65% of the total c.f.u. were represented by the water administered strain, while the corresponding figure for the orally administered strain was 55%. Day 7, the water administered strain represented 10% of the total c.f.u. and 45% the orally administered (Olsson et al., unpublished studies). No streptomycin resistant strains were found in the control.

The continuation of this project includes coculturing of the pathogen and a probiotic strain in fish and an attempt to describe the port of entry of the pathogen in the fish. To be able to do this a highly sensitive detection system is needed. One alternative is a method which uses the specificity in the sequence of the 16 S RNA molecule. One of the sequences was found suitable to use as a fluorescent-labelled probe for in situ detection using epifluorescence microscopy. The probe sequence was; 5'-TTG GCC CGT AGG CAT C-3', responding to the 16 S rRNA sequence of *V. anguillarum* position 180-196 [19]. Using this method it was possible to detect single cells of *V. anguillarum* in intestinal mucus from turbot infected with the pathogen (Westerdahl et al., unpublished studies). It can be concluded that constituents of the indigenous microflora of the turbot intestine can be inhibitory to *V. anguillarum* in vitro. It is feasible to propose that administration of some of these isolates could protect farmed turbot from infection.

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PHENOMENON OF RICKETTSIELLA PHYTOSEIULI IN PHYTOSEIULUS PERSIMILIS MITE*

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An unknown microorganism occurring in a predaceous mite *Phytoseiulus persimilis* was described by the author in 1977 as a new species *Rickettsiella phytoseiuli*. Some new results on the relation between this agent and its hosts are presented in this paper.

The predatory mite, *Phytoseiulus persimilis*, has been used in many countries for several years to control the noxious spider mites of the genus *Tetranychus* in greenhouse cultures. In 1974 we performed an electron microscopy (EM) study of the ultrastructure of the digestive system of this predatory mite in relation to its physiological capacity to ingest considerable amounts of food (spider mites) within short periods of time. However, we had to abandon our original intention, as all mites were found to be infected with an unknown rickettsia-like organism of genus *Rickettsiella* later described as a tentative new species, *R. phytoseiuli* [1].

So far, rickettsia-like organisms have been described from *Metaseiulus* (= *Typhlodromus*) *occidentalis* [2]. In *P. persimilis*, unidentified bacteria were found in the lumen of its alimentary tract [3]. Having accidentally found a repeated rickettsial infection in *P. persimilis* in 1985, we returned to this problem, stimulated by the extent of the infection as well as the fact that *P. persimilis* examined by Arutunyan [3], was not found to be infected with rickettsiellae.

This review is a brief summary of our findings during 1974–1977 and 1985–1991 in order to demonstrate this extraordinary events in the relationship between microorganism and host.

Materials and methods

Adult mites of P. persimilis were obtained from three laboratory cultures. The mites showed no external abnormalities, morphological changes or increased mortality. Origin of mite colonies: 1.

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1974–1975, 1989–1991 – Institute of Zoology, Academy of Sciences of the Ukrainian Republic, Kiev, Ukrainian Republic. 2. 1985–1989 – Institute of Experimental Phytopathology and Entomology, Slovak Academy of Sciences, Ivanka pri Dunaji, Slovakia. 3. 1986 – Institute of Zoology, Academy of Sciences of the Armenian Republic, Yerevan, Armenian Republic.

All *P. persimilis* mites were reared on *Tetranychus urticae* mites, which in turn, were fed in laboratory conditions on bean plants.

Developmental stages of *P. persimilis* (larvae, 1st and 2nd nymphs) were studied in Materials 1 (1989, 1990) and 2 (1986).

For the cultivation of rickettsiellae, after successfully isolation, the half-engorged ticks, *Dermacentor reticulatus* were utilized. These ticks were collected in flooded forest areas near Bratislava. The methods of isolating *R. phytoseiuli* from *P. persimilis* mites and the application to *D. reticulatus* ticks were described in a previous paper by Šutáková and Řeháček [4].

Detailed procedures for preparation of the material for EM are described in papers by Šutáková [5], Šutáková and Řeháček [4] and Šutáková and Arutunyan [6].

Results and discussion

In the course of our study the following achievements and new results were obtained.

1. Six morphologically well-differentiated cells of rickettsiella were found representing developmental stages: dense, intermediate, bacterial, giant, crystal-forming and small dark particles. These stages were found in large amounts in all tissues except the nervous system of the adult *P. persimilis* [1, 5].

2. A complete description of the developmental cycle of rickettsiella is presented in its natural host, *P. persimilis*. We consider small dark particles as the first developmental phase. When the small dark particles are released from the crystal-forming rickettsiae, they grow, gain their membrane complex and change in this way to the dense type of rickettsia. These are transformed to intermediate and later to the bacterial type of rickettsiae. The bacterial types of rickettsiae can multiply by binary fission. Some bacterial cells transform to giant. These form a crystal inside and they are converted to crystal-forming types of rickettsiae in which the above-mentioned small dark particles had already developed. For the genus *Rickettsiella* no such reproductive cycle has been reported. We propose to name this rickettsia as *Rickettsiella phytoseiuli* [1, 5].

3. All developmental stages (larvae, protonymphs, deutonymphs) from all laboratory cultures were tested for infection with *R. phytoseiuli*, but as yet have not found to be infected [5, 6].

4. We have been successful in both the isolation of *R. phytoseiuli* cells from the natural host and their subsequent cultivation in the ticks *D. reticulatus* as experimental hosts. *R. phytoseiulus* demonstrated all six developmental stages as in its natural host [4, 7].

5. We have described the distribution of *R. phytoseiuli* cells in the experimental host and defined the sites of their predominant localization. *R. phytoseiuli* was found in salivary glands, Malpighian tubules, ovaries, tracheal complex, haemolymph, fat body and the alimentary tract. *R. phytoseiuli* did not infect the Gene's organ. The predominant localization is in the fat body, cortical layer of the synganglion and tracheal complex [7].

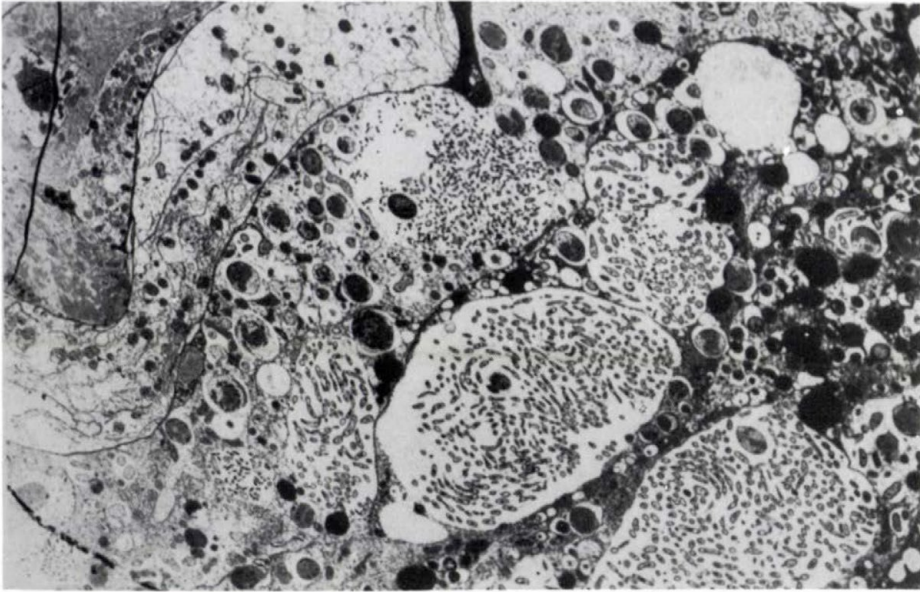


Fig. 1. Massive infection of *P. persimilis* female by all stages of *R. phytoseiuli*. Ukrainian material, Kiev, 1975.
Bar=4 μ m

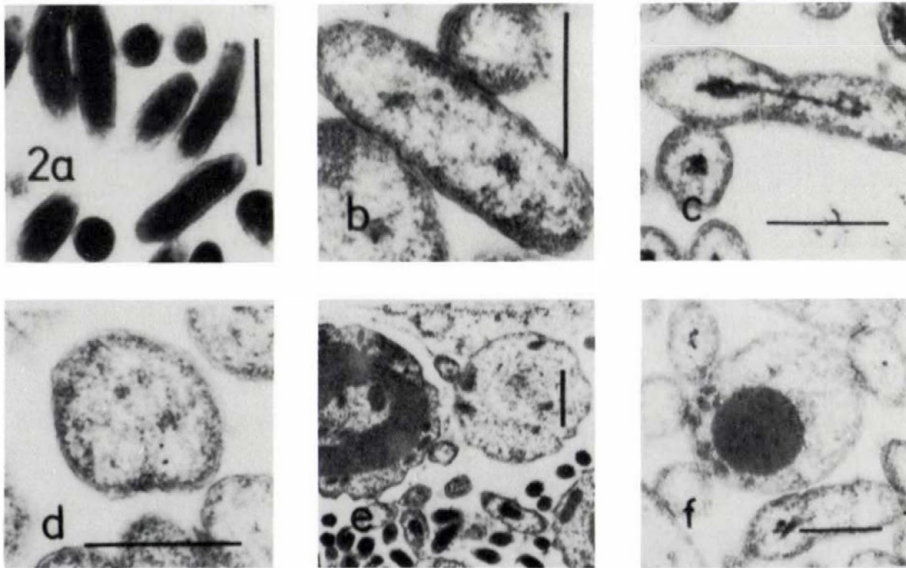


Fig. 2. Morphologically well-differentiated cells of *R. phytoseiuli*: dense (a); intermediate (a, e); bacterial (b, c, f); binary fission of bacterial rickettsia (c); giant (d); crystal-forming with small dark particles (e, f).
Czechoslovak material, Ivanka pri Dunaji, 1986. Bar=500 nm

6. The existence of dual experimental infection (with *R. phytoseiuli* and *Coxiella burnetii*) in the experimental host, *D. reticulatus* ticks was demonstrated. Following intracoelomic infection, both agents, with the exception of Gene's organ, multiplied well in the cells of the tick host's organs. Two out of six developmental stages of *R. phytoseiuli*, i.e. crystal-forming and small dark particles, in dual infection with *C. burnetii* revealed marked morphological alterations, and demonstrated the importance of the interaction between both pathogens [8].

7. Records were kept of the occurrence of natural infections of the mite *P. persimilis* with virus and *R. phytoseiuli*, pointing out their mutual interaction [9].

8. The description of the presence of symbiotic microorganisms in uninfected *P. persimilis* was presented [6].

The difference between the presence *R. phytoseiuli* in mites and in the described rickettsiellae in insects and spiders is based on the fact that the mite *P. persimilis* (and ticks *D. reticulatus*) remained alive even after massive infection while the infection in insects and spiders was lethal [9–12]. In insects and spiders the fat body and haemolymph were primarily infected. The infected individuals died usually during the larval stage.

The infection of the mite *P. persimilis* with *R. phytoseiuli* as described above represents an unusual phenomenon in relationships between the microorganism and its host. In future studies it will be necessary to specify the character of the relationship between *R. phytoseiuli* and its hosts and focus attention to determining the source of infection of *P. persimilis* with rickettsiellae. A possible infection by *R. phytoseiuli* from this pest has not been confirmed. Accordingly on this phenomenon could have great importance in biological control in agriculture.

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PENAEID PRAWNS AND ASSOCIATED LUMINOUS BACTERIA*

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Luminous bacteria associated with the exoskeleton, gill and gut of penaeid prawns, *Penaeus indicus* H. Milne Edwards and *Penaeus monodon* Fabricius in Mangalore waters were isolated, identified and quantified. Two species of bacteria, *Vibrio harveyi* and *Vibrio fischeri* were recorded, the former was dominant in the exoskeleton and gill of both the penaeids. Gut of prawns supported exclusive and dense populations of *V. harveyi* suggesting that the species is well adapted to proliferate in this microenvironment especially in the hindgut region.

Luminous bacteria are ubiquitous in seawater occurring as freeliving, saprophytic, symbiotic and parasitic forms [1-7]. They also occur in estuarine sediments [8]. The association of luminous bacteria with marine and estuarine organisms is attributed mainly to the availability of increased concentrations of nutrients and other organic materials in the hosts, essential for the growth [9]. Few species of luminous bacteria cause mass mortalities of prawn larvae in the hatcheries [10]. However, information on the association of luminous bacteria with the feral prawns from India is scanty [11, 12]. Hence, the present study was undertaken to provide information on the distribution profiles of luminous procaryotes harboured on the exoskeleton and gill, and in the gut of the host prawns.

Materials and methods

For the isolation of the luminous bacteria, live prawns belonging to the species *Penaeus indicus* H. Milne Edwards and *P. monodon* Fabricius were obtained from the trawl caught off Mangalore (Lat. 12°52' N and Long. 74°45' E) in the Arabian Sea during March, 1991. The prawns kept in the holding tanks were transported to the laboratory and sacrificed immediately. Swabs obtained aseptically from the surfaces of exoskeleton and gill of prawns were serially diluted and plated in duplicate on the complex seawater agar (SWC) medium [3] following spread plate technique.

*Based on presentation at the "Second International Colloquium on Microbiology in Poikilotherms" organized by the Hungarian Academy of Sciences and L. Eötvös University, Budapest, June 15-17, 1992

To isolate the enteric gut flora, the gut of the prawn was removed aseptically and divided into foregut (oesophagus and stomach), midgut (mesenteron) and hindgut (rectal region). The three gut sections were weighed and homogenized individually in 100 ml sterile seawater. Serial dilutions of the homogenate were made and plated in duplicate onto SWC agar medium.

After 36 h of incubation at room temperature (28 ± 2 °C), the total colony forming units (T.c.f.u.) were counted in light and the luminous colony forming units (L.c.f.u.) in dark. The luminous colonies were picked up at random from the culture plates and maintained in SWC agar slants for further characterisation of species [13]. The bacterial population densities were calculated for cm^2 area of exoskeleton and g wet weight of gill and gut content. Two-way analysis of variance technique was used to test for the significant difference in the average luminous bacterial load due to different regions and lengths of the prawns.

Results and discussion

In *P. indicus*, gut harboured higher number of luminous bacteria than the exoskeleton and gill (Table I). While the luminous microfloral count fluctuated from 9.3×10^2 to 7.9×10^3 c.f.u./ cm^2 in the exoskeleton and 8.2×10^2 to 7.6×10^3 c.f.u./g wt. of gill, it was between 4.3×10^5 and 5.3×10^6 c.f.u./g wt. of gut content. The luminous bacteria contributed to 13.8 to 56.7% of the total heterotrophic bacterial population in the exoskeleton, 28.9 to 71.4% in gill and 51.3 to 78.1% in the gut (Table II).

Table I

Density of luminous bacteria in different regions of *Penaeus* spp.

Species	Length (mm)	Bacterial density		
		Exoskeleton (Count/ cm^2)	Gill (Count/g wet wt.)	Gut (Count/g wet wt.)
<i>P. indicus</i>	88	2.6×10^3	5.1×10^3	8.4×10^5
	96	1.9×10^3	2.5×10^3	5.3×10^6
	101	7.9×10^3	8.2×10^2	3.9×10^6
	109	9.3×10^2	7.6×10^3	1.2×10^6
	133	4.4×10^3	5.9×10^3	4.5×10^5
	146	6.2×10^3	6.6×10^3	3.3×10^6
Average		3.16×10^3	3.82×10^3	1.77×10^6
<i>P. monodon</i>	158	1.3×10^3	6.5×10^3	2.4×10^6
	165	1.6×10^3	7.2×10^3	1.5×10^5
	176	5.3×10^3	5.6×10^3	3.1×10^6
	185	8.1×10^3	3.1×10^4	2.9×10^6
	195	6.3×10^3	4.6×10^3	5.9×10^6
	225	2.4×10^4	2.3×10^3	7.1×10^5
	237	2.9×10^3	1.9×10^4	8.6×10^5
Average		4.53×10^3	7.72×10^3	1.42×10^6

Table II

Percentage of L.c.f.u. among T.c.f.u. associated with *Penaeus* spp.

Species	Length (mm)	Region		
		Exoskeleton	Gill	Gut
<i>P. indicus</i>	88	28.3	40.5	62.6
	96	44.4	39.8	51.3
	101	41.2	28.9	64.5
	109	13.8	45.5	78.1
	133	33.4	49.2	68.2
	146	56.7	71.4	70.8
<i>P. monodon</i>	158	36.0	52.6	69.4
	165	62.7	44.2	72.8
	176	28.1	21.8	55.6
	185	53.9	37.4	48.2
	195	40.6	40.3	45.5
	225	35.1	30.5	34.7
	237	44.2	38.7	39.3

The gut of *P. monodon* also supported dense populations of luminous bacteria as compared to the exoskeleton and gill (Table I). The luminous bacterial count ranged from 1.3×10^3 to 2.4×10^4 c.f.u./cm² in exoskeleton, 2.3×10^3 to 3.1×10^4 c.f.u./g wt. of gill and 1.5×10^5 to 5.9×10^6 c.f.u./g wt. of gut content. The percentage of luminous isolates in the T.c.f.u. ranged from 28.1 to 62.7% in the exoskeleton, 21.8 to 52.6% in the gill and 34.7 to 72.8% in the gut (Table II). The results of statistical analysis of luminous bacterial load in both the species (after logarithmic transformation) show that there is a high significant difference in the average density of luminous bacteria due to regions (exoskeleton, gill and gut) ($p > 0.05$) but not due to length of prawns (Table III).

Although, three species of luminous bacteria viz., *Vibrio harveyi*, *V. fischeri* and *Photobacterium leiognathi* have been reported from the Mangalore region [14], only the former two species were found in association with penaeids in the present study (Table IV). While *V. fischeri* occurred in lesser numbers in exoskeleton and gill, *V. harveyi* was most dominant in both exoskeleton and gill and was also exclusively present in the foregut, midgut and hindgut regions of *Penaeus*. The percentage contribution of *V. harveyi* to the total luminous isolates obtained from *P. indicus* was 96.2% and from *P. monodon* was 96.4%. Though, *P. leiognathi* could not be isolated from any of the prawn microenvironments presently, it was reported from the cuticle and gut of the crabs of Porto Novo waters [12].

Table III

ANOVA table on the density of luminous bacteria in different regions and lengths of *Penaeus* spp.

Source of variation	Species	Mean sum of squares	F-ratio
Due to regions	<i>P. indicus</i>	14.6758	78.7033*
	<i>P. monodon</i>	13.3170	66.0480*
Due to lengths	<i>P. indicus</i>	0.0641	0.3436
	<i>P. monodon</i>	0.2228	1.1050
Error	<i>P. indicus</i>	0.1865	
	<i>P. monodon</i>	0.2016	

* Significant ($p > 0.05$)

Table IV

Species composition of luminous bacteria isolated from *Penaeus* spp.

Species	Region	No. of luminous isolates tested	Percentage	
			<i>V. harveyi</i>	<i>V. fischeri</i>
<i>P. indicus</i>	Exoskeleton	20	95.0	5.0
	Gill	24	91.7	8.3
	Gut			
	i) Foregut	12	100.0	—
	ii) Midgut	5	100.0	—
	iii) Hindgut	18	100.0	—
<i>P. monodon</i>	Exoskeleton	25	92.0	8.0
	Gill	19	94.7	5.3
	Gut			
	i) Foregut	12	100.0	—
	ii) Midgut	10	100.0	—
	iii) Hindgut	18	100.0	—

Among the foregut, midgut and hindgut regions of penaeids of both the species that supported dense populations of *V. harveyi*, the percentage composition of these bacteria was the highest in the hindgut followed by the foregut and the midgut (Table V). The higher incidence of luminous microflora in the hindgut has also been reported from prawns and crabs of Porto Novo [11, 12].

While there was significant difference in the average luminous bacterial load in different lengths and gut regions of both the species of prawns ($p > 0.05$), the difference was more pronounced in *P. monodon* (Table VI). However, it was found that the

average luminous bacterial load was not influenced by the lengths of prawns in the exoskeleton, gill and gut.

Table V

Percentage of V. harveyi in different gut regions of Penaeus spp.

Species	Length (mm)	Foregut	Midgut	Hindgut
<i>P. indicus</i>	88	18.0	6.3	75.7
	96	39.8	12.1	48.1
	101	28.3	22.0	49.7
	109	33.5	20.8	45.7
	133	40.4	16.1	43.5
	146	24.3	21.0	54.7
<i>P. monodon</i>	158	22.2	12.5	65.3
	165	31.4	16.8	51.8
	176	18.6	9.5	71.9
	185	30.9	11.3	57.8
	195	24.1	20.7	55.2
	225	18.0	14.6	67.6
	237	25.7	15.2	59.1

The dominance of *V. harveyi* in the exoskeleton and gill, and its selective presence in the gut of prawns indicate that this species is nutritionally more versatile than *P. fischeri*, as the former is known to utilize 30–45 organic compounds as nutrients and the latter only 7–22 organic compounds [15]. Further, the existence of heavy population of *V. harveyi* in the gut shows its less sensitivity to the digestive enzymes and adaptability to the conditions prevailing in this microenvironment especially the faecal matter in the hindgut that would serve as an ideal inoculum for proliferation of this bacterium. It is also postulated presently that *V. harveyi* may also find its way through the anal region of the prawns to colonise the hindgut. Apart from nutritional capabilities, the presence of lateral flagella of *V. harveyi* is expected to provide not only enough surface area for adsorption in the prawn microenvironments like exoskeleton, gill and gut, but also, inhibits the growth of polarly flagellated bacteria like *V. fischeri* and *P. leiognathi* [16, 17].

The lesser luminous bacterial density in the exoskeleton and gill than in gut might be due to the periodic moulting of prawns. Further, the constant exposure of exoskeleton and gill to the movement of surrounding water might also limit the saprophytic luminous bacterial population. As the prawns are detritus feeders and bottom dwellers, the ambient water and sediment may serve as the sources of luminous bacteria to colonise the prawns [4, 5, 18]. However, the physico-chemical conditions of

the host body would play a role in the selection and maintenance of commensal flora of the marine organisms [19].

Table VI

ANOVA table on the abundance of *V. harveyi* in different gut regions and lengths of *Penaeus* spp.

Source of variation	Species	Mean sum of squares	F-ratio
Due to gut regions	<i>P. indicus</i>	0.7654	12.8776*
	<i>P. monodon</i>	0.7329	73.8438*
Due to lengths	<i>P. indicus</i>	0.5471	9.2011*
	<i>P. monodon</i>	0.8322	83.9446*
Error	<i>P. indicus</i>	0.0595	
	<i>P. monodon</i>	0.0099	

*Significant ($p > 0.05$)

Though *V. harveyi* is not pathogenic to prawns in the wild, under certain unfavourable hatchery conditions, it becomes an opportunistic pathogen causing mass mortalities of prawn larvae [10]. This shows that *V. harveyi* is cosmopolitan in distribution and can thrive in freeliving, saprophytic, enteric and parasitic niches.

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ENDOTOXINS AND PREDNISOLONE ALTER REPLICATION OF TYPE 5 ADENOVIRUS AND ITS TEMPERATURE SENSITIVE MUTANTS*

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Latency, replication or transformation by adenoviruses require cooperation between their gene products and cellular factors, which are controlled by external stimuli. Clinical observations suggest that bacterial endotoxin (LPS) and steroid hormones have direct effects on the viral permissivity and activation. Therefore, HEp-2 cultures were infected with low multiplicity of wild type (WT) human adenovirus 5 (Ad-5) and its temperature-sensitive mutants *ts18* and *ts19* damaged in the phosphorylation of structural polypeptides VI and X at 39 °C, respectively. Cultures were treated at permissive (32 °C) and nonpermissive (39 °C) temperatures with native and radio-detoxified (RD) LPS, α -tocopherol and prednisolone alone or in combination. Titration of virus yields showed dose-dependent activation and enhanced replication of latent WT and *ts* mutants by both LPS preparations. α -Tocopherol diminished these processes. LPS was likewise unable to augment virus producing capacity of cells. Prednisolone, although activating no latent virus, resulted in augmenting Ad-5 production at 32 °C. No compounds activated mutants at 39 °C except synergistic effect of LPS and prednisolone resulted in limited but significant replication of mutant *ts19*. Deliberation of lysosomal enzymes, enhanced cGMP and tumour necrosis factor α (TNF- α) productions by LPS as well as interaction of Ad early gene expression with prednisolone-utilized cellular transcription factors including AP-1 (*c-jun*, *c-fos*) are implicated in these processes. Excluding the role in activation of Ad proteinase processing viral structural polypeptides draws attention to the importance of cellular factors in virus replication.

*To the memory of Professor Alois Nowotny, 1922, Gyöngyös, Hungary – 1991, Philadelphia, USA.

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Programming cells to support pathogenic processes by adenoviruses (Ads) starts at the moment of adsorption of virus particles to cellular surface and continues during both their directed transport towards nucleus and uncoating, before any viral gene would have been expressed. In the next phase, expression of the early (E) genes requires utilization of several transcriptional host factors, whose regulation by phosphorylation is under the control of secondary messenger system of cells [1, 2]. The interaction between early viral gene products and available host factors determine the fate of infection, namely, latency, transformation or productive infection [3–7]. If the last one is “chosen”, cellular machinery supports production of viral DNA and protein synthesis, as well as assembly and release of particles during the so-called late phase of virus replication instead of cellular DNA and protein synthesis which become completely inhibited [8, 9]. All phases of adenoviral life cycle, including the sequential expression of early and late genes, and the outcome of infection largely depend on the origin and type of cells, and their actual state in the cell cycle [1, 4, 10–12]. In the host, expression and quantity of several cellular factors playing a role in viral metabolism are under control of external stimuli (hormones, cytokines, prostaglandins /PGs/, etc.), whose majority acts through receptor mediated secondary messengers [6, 13, 14]. Endotoxins of Gram-negative bacteria are able to modify cellular metabolism [2]. The pathogenic effects of Ads depend on partial or complete activation by heterologous effects. The majority of strains productively infects epithelial cells because of their permissivity [17–19]. Those belonging to Group C (types 1, 2, 5, 6) establish latent infection in lymphocytes because of their nonpermissivity [4, 15, 17, 20]. Upon activating by mitogens *in vitro* or *in vivo*, blastogenic transformation of these lymphocytes confers semipermissivity with limited replication [4, 18]. *In vivo* such phenomenon results in reappearance of clinically manifested infection. Several human adenovirus types establish abortive infection in rodents, of whom members of Group A (types 12, 18, 31) by expressing their E1A and E1B genes transform and immortalize cells causing tumours [11, 21–23]. Group C serotypes by their E1A gene product cause immortalization, but complementing expression of *Ha-ras* gene results in transformation and tumorigenicity [1, 9, 21, 23]. Their possible role in human tumours involving nonpermissivity in lymphocytes [24–26] is discussed elsewhere [27]. Depending on serotypes, expression of some E1 and E3 genes results in avoiding immune surveillance. Infection modulates several immune functions, too [9, 15–17, 19, 22, 28–30].

Depending on serotypes, expression of some E1 and E3 genes results in avoiding immune surveillance of infected cells, which also contributes to tumorigenicity [15, 17, 28]. Expression of the same early genes affects the production of interferon (IFN) and other cytokines [17, 19, 29], natural killer (NK) cell activity [22] and several immune functions [9, 22]. Even nonpermissive infections can modulate early gathering of phagocytic cells [16, 28], suppress phagocytic functions but promote bacterial growth via soluble factors parallel to their oncogenic potential [30]. It is known that, the transcriptional control regions of the Ad early units E1A, E2, E3 and E4 contain cAMP responsive elements (CRE), which share properties with cellular CREs, and can specifically bind a cellular transcription factor, CRE-binding protein (CREB) [2]. Members of several other families (AP-1, ATF/CREB, etc.) have been implicated in mediating transcriptional activation effect of E1A and cAMP, which are synergistic [23, 31]. We have shown recently that in the case of oncogenic

and latent serotypes both infection process itself (entry, transport) and the late phase leading to particle assembly in HEP-2 cultures are enhanced by $\text{PGF}_{2\alpha}$, which is known to activate cGMP. These latter processes are diminished by PGE_2 , known to activate cAMP [14]. At restrictive temperature, limited reactivation of a temperature sensitive (*ts*) phosphorylation mutant (*ts18*) of Ad-5 suggested that a cGMP dependent protein kinase (PK) has a role in these processes. Non-oncogenic adenovirus showed less sensitivity to the effects of PGs. Latent Ad infections were activated by microbial products [32], of which LPS interacted with cells [33], and by prednisolone. Activation was characterized by appearance of antigens prior to cytopathic effect (CPE) [34]. LPS also augmented transcription of woodchuck hepatitis virus (WHV) [35] and human hepatitis B virus (HBV) [36] in peripheral blood mononuclear cells (PBMC).

LPS facilitates intracellular cGMP production [37, 38] counteracting PGE_2 and cAMP mediation [39, 40] and activates phospholipase A_2 [41], which regulate cyclic nucleotide (CN) dependent and independent protein kinases (PKs) [14, 42]. It binds to receptors of several immune [43, 44] and non-immune cells [45, 46] resulting in altered protein phosphorylation and cytoskeletal breakdown [47]. LPS also induces toxic oxygen [44] and nitrogen [48] radicals, damages both plasma and lysosomal membranes consequently deliberating lysosomal enzymes [49]. Noxious effects of LPS can be reduced by detoxification procedures, of which irradiation proved to be one of the most successful. The resulting radiodetoxified LPS (RD-LPS) retains several beneficial effects [33, 49, 50]. Certain influences of LPS and PGs are antagonized by α -tocopherol (Vitamin E), that prevents formation of toxic oxidation products [51], stabilizes biological membranes [52], and promotes cellular DNA synthesis [53].

Steroid hormones diffuse passively through cell membranes, distribute themselves throughout the cell and ultimately bind to the mineralocorticoid, glucocorticoid, progesterone and androgen receptors (MR, GR, PR, AR, respectively) [54]. The hormone-receptor complex moves into the nucleus, where the central domain of the receptor binds to hormone responsive elements (HREs, designated originally as glucocorticoid responsive elements, GREs) in the promoter region of genes, whose transcription is controlled by a given steroid [2, 55]. Their transcription is enhanced, or rarely, decreased [51]. All nucleated cells contain GR, and the specificity of hormone action is explained by additional cellular mechanisms [55]. Estrogens utilize other responsive elements [91]. Interactions of steroid receptors with other transcription factors are explained that both the steroid receptors and transcription factor Ap-1 can bind to a 25 base pair (bp) composite responsive element (called *plfG*) [55]. The subunit components of AP-1, *c-fos* and *c-jun* proto-oncogene products are expressed in response to a variety of stimuli – including Ad E1A and cAMP [31, 23] –, and their ratio determines transcriptional activation or inhibition by different steroids [55]. ACTH stimulating corticoid and androgenic substances increases cAMP [51].

Replication, latency or transformation by several viruses, such as herpes simplex virus type 1 and 2 (HSV-1, -2 [56, 57]), bovine HSV-1 [58], human papillomaviruses (HPV [59–63]), adenoviruses [32, 34, 64–67], human immunodeficiency virus type 1 (HIV-1 [68–71]), mouse mammary tumour virus (MMTV [72, 73]), etc. can be influenced by natural and synthetic steroid hormones. Retroviruses, including HIV-1 [68] and MMTV [55, 73] contain GRE/HRE in their long terminal repeat (LTR) sequences, and different HREs were found in oncogenic types of HPV

[59, 60, 62]. The hormone sensitivity of other viruses could be due to either direct or indirect effects inside cells, or due to immune modulation *in vivo*. Glucocorticoids inhibit synthesis and/or release of arachidonic acid and its metabolites (PGs, LTs), tumour necrosis factor α (TNF- α), interleukin 1 (IL-1) and IL-2 [51].

Interactions between LPS and hormones have been known. LPS stimulates the hypothalamic-pituitary-adrenal (HPA) axis to release ACTH and cortisone [74], while exogenous glucocorticoids such as dexamethasone protect mice against lethal effects of LPS by down-regulating its TNF- α production [75].

We carried out detailed studies on the activation by LPS and prednisolone as well as their combination of latent prototype Ad-5 WT. As virus mutants are also used successfully in modelling latency [9, 15, 16, 28, 29, 76], these studies were extended to two *ts* mutants with known defects in polypeptide phosphorylation [14, 77]. Immunological mechanisms were excluded by infecting a conventional, permissive tissue culture with low multiplicity of virus inocula.

Materials and methods

Cells and viruses. HEp-2 cells (obtained from the National Institute of Health, Bethesda, MD, USA) were maintained by usual trypsinization weekly [32, 33]. Their growth medium consisted of Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS, Phylaxia, Budapest, Hungary), 0.075% NaHCO_3 , 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin, while maintenance medium contained only 2% FCS. Adenovirus wild type 5 (Ad-5 WT, obtained from Dr.H.G.Pereira, London, UK) was grown at 37 °C, while its two mutants *ts18* and *ts19* (obtained from Dr.B.Taródi, Szeged, Hungary [78]) were grown at permissive temperature (32 °C) in HEp-2 cultures by a standard procedure [33], and partially purified [30]. WT was titrated at 37 °C, but mutants were titrated at both permissive and restrictive (39 °C) temperatures [78]. For synchronized infections, 1 tissue culture dose (TCID₅₀) was determined by interpolation, which unit equals to a minimal, but surely cytopathic dose for all cultures tested at the same time [14, 33, 78]. Its use instead of the usual TCID₅₀ considered because small amounts of viruses were used throughout the experiments. Viruses were stored in 1 ml aliquots at -20 °C and diluted accordingly to TCID₅₀ calculations.

Treatment and infection regimen of tissue cultures. Endotoxin (LPS) was extracted by the phenol-water method of Westphal from the fermentor culture of *E. coli* 089 and purified by repeated ultracentrifugation and quantitated subsequently [79]. One part of this native LPS was then suspended in distilled water (10 mg/ml) and was irradiated to be detoxified by 150 kGy (⁶⁰Co-Gamma, Noratom 3500) [80]. Both native LPS and the radiodetoxified form (RD-LPS) were stored at -20 °C. α -Tocopherol was obtained from Sigma (St. Louis, MO, USA), and 21-prednisolone succinic acid sodium salt (DiAdreson F aquosum) was purchased from Organon (Oss, The Netherlands). All compounds were diluted with maintenance medium immediately before use. Their concentrations which are nontoxic and do not influence cell growth had been previously determined [32, 33]. 2×10^5 HEp-2 cells in 1 ml growth medium were aliquoted into test tubes. Upon reaching confluency, their medium was replaced by fresh one containing 2% FCS and dilutions of virus stocks. Parallel cultures were incubated at 32 °C and 39 °C and their medium was supplemented with 0.1 ml of compounds at final concentrations indicated in the Table and Figures at different times between days 1 and 10 (referred to as "late treatment"). In certain treatment regimens, LPS and α -tocopherol or LPS and prednisolone were added to the same cultures simultaneously. To study their effect of pretreatment on virus infections, medium of confluent cultures were replaced with one containing 2% FCS and compounds at indicated concentrations, then 24 or 48 h later their medium was supplemented with virus dilutions in 0.1 ml volume. Mock treated and mock infected cultures served as controls. All tests were prepared in 6 parallel samples. Cultures were incubated at the same temperature; or in the case of

mutants, some of parallel ones were shifted to another temperature. At different time intervals, 0.1 ml aliquots of supernatant fluids were removed for virus titration as described above. In several occasions, detached cells were subjected to indirect immunofluorescence (IF) to determine their virus antigen content [32].

Results

Effect of LPS and α -tocopherol on adenovirus strains. For a limited period depending on the viability of cultured cells, latent state could be established at both temperatures using small inocula (10^{-2} – 10^{-5} TCID₅₀) of wild type adenovirus, but higher inocula (10^{-1} – 10^1 TCID₅₀) resulted in efficient virus replication (Fig. 1: A, D). At the permissive temperature behaviour of mutant *ts18* (Fig. 2: A, D) and mutant *ts19* (Fig. 4: A) were identical to the wild type, but at restrictive temperature no replication of mutants took place, even if higher inocula were used. In any form of nonpermissive infection, however, virus absorption took place and around 0.5% of cells contained viral antigens detected by IF. Native LPS in a dose dependent manner could induce replication of WT at both temperatures, but it was not able to enhance virus producing capacity of cells if higher inocula were used (Fig. 1: B, D). During cultivation, latent virus could be activated at any time, as it is shown in the case of mutant *ts18* at permissive temperature (Fig. 2: B, E, F and Fig. 3: E, F). The activating effect of RD-LPS in a dose dependent manner was similarly effective at any time postinfection at both temperatures in the case of WT (Fig. 1: C, F) and at permissive temperature in the cases of mutants (Fig. 2: C and Fig. 4: B, C). Pretreatment of cells with either LPS or RD-LPS prevented establishing latent state by WT at both temperatures and at permissive temperature by mutants, as the latter is shown (Fig. 5: E). Temperature shift from permissive to the restrictive resulted in an immediate cease of replicating mutants (Fig. 3: D and Fig. 5: D). If small inocula of mutants had been activated previously by endotoxins at permissive temperature, subsequent shift to nonpermissive temperature at any time inhibited further virus production (Fig. 3: E, F and Fig. 5: F). At restrictive temperature cells were susceptible for infection as IF revealed virus antigens in them. Higher inocula of mutants induced replication after shifting cultures to the permissive temperature (Fig. 3: A and Fig. 5: A). Presence of endotoxins was not able to activate latent viruses, only after shifting cultures to permissive temperature (Fig. 3: B, C and Fig. 5: C). Appearance and distribution of virus antigens shown preceded CPE by one day approximately.

α -Tocopherol was not able to reactivate latent adenovirus infections at any temperature, and in a dose dependent manner it decreased replication of all virus strains tested. Treatment of cell cultures prior to infection rendered them less sensitive to support virus replication, as proved by the moderate slope of replication curves (data not shown). Similarly, pretreatment of cells significantly inhibited both reactivation process and enhanced replication by endotoxins, but adding α -tocopherol to the cells, which had been infected previously, could inhibit the stimulatory effect by endotoxins in a smaller degree (Table I). Decay of α -tocopherol added to cultures prior to infection made reactivation and enhanced virus production by endotoxins possible gradually (data not shown).

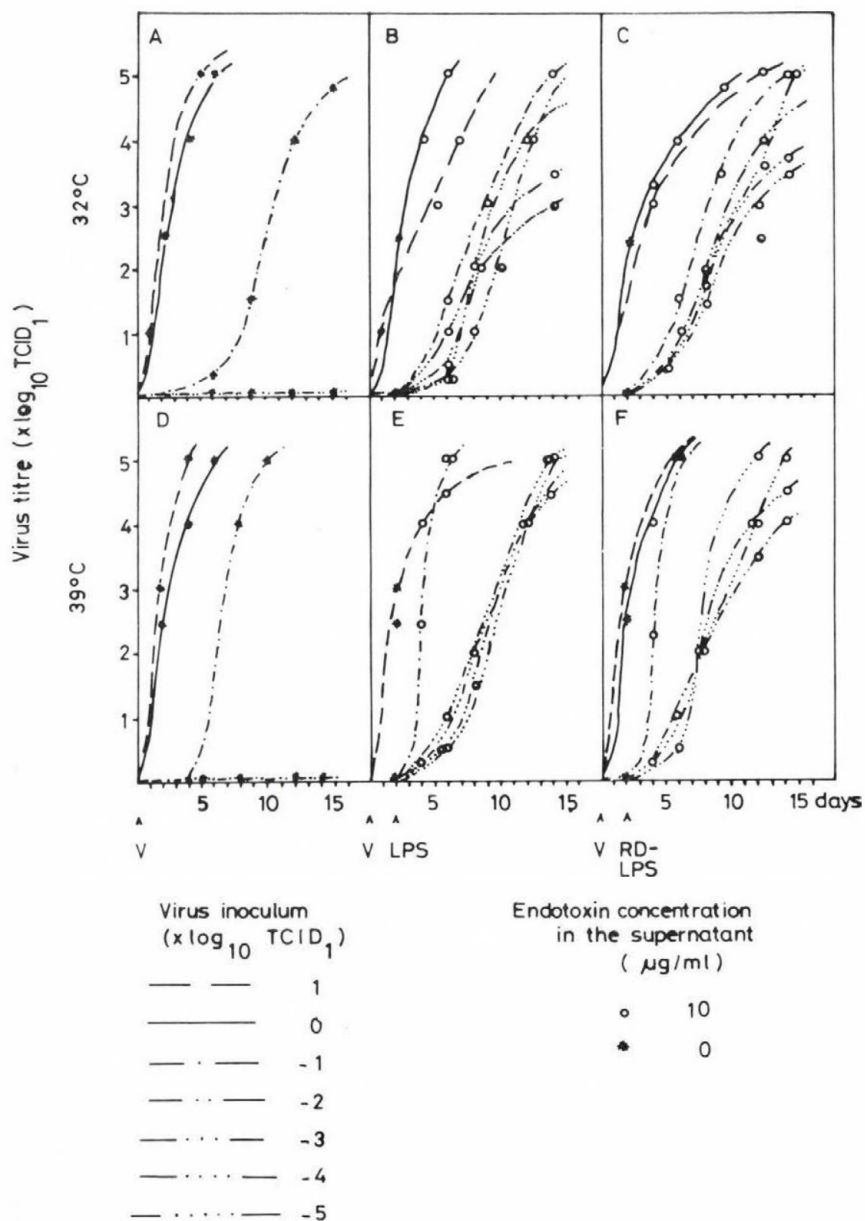


Fig. 1. The effect of endotoxins on the replication of Ad-5 WT

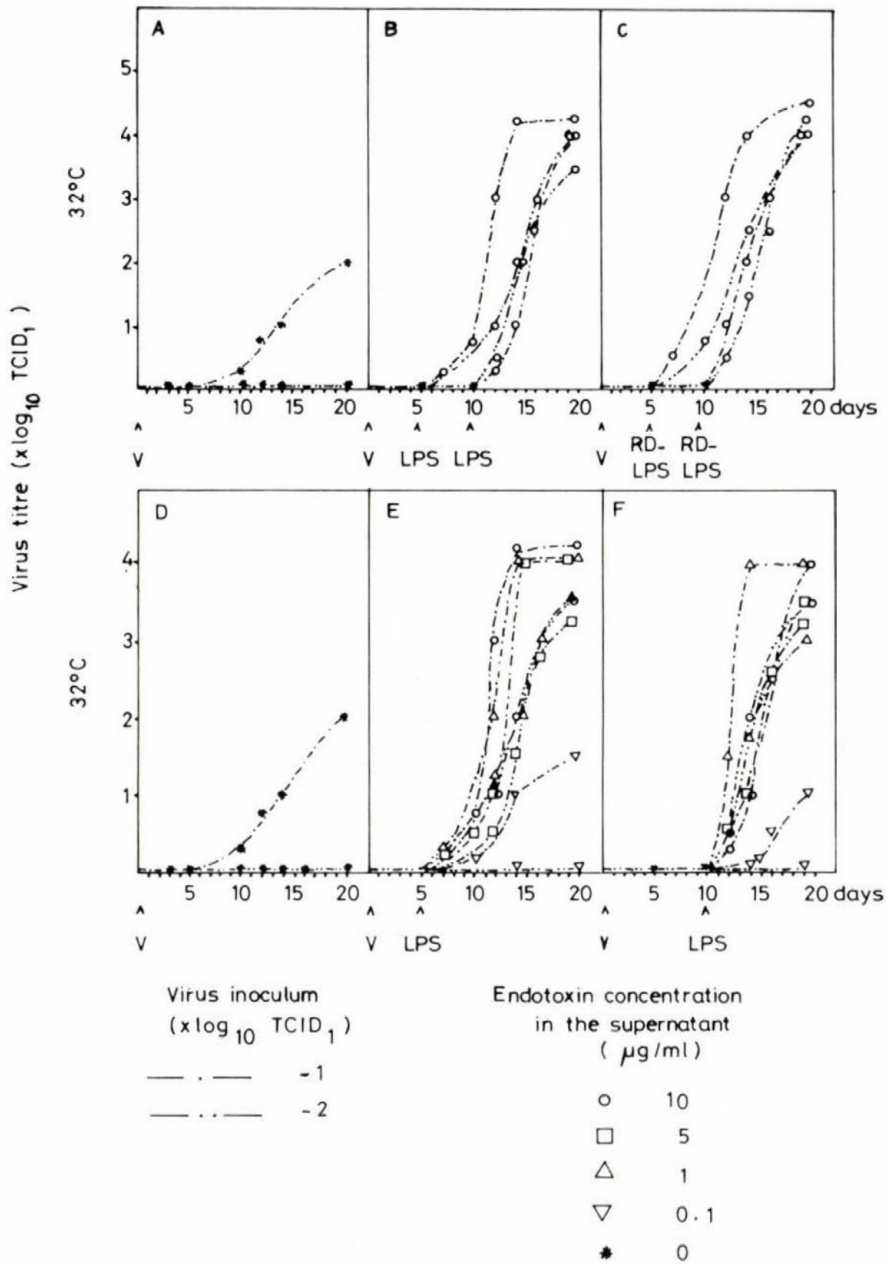


Fig. 2. The activating effects by LPS and RD-LPS on Ad-5 mutant *ts18*

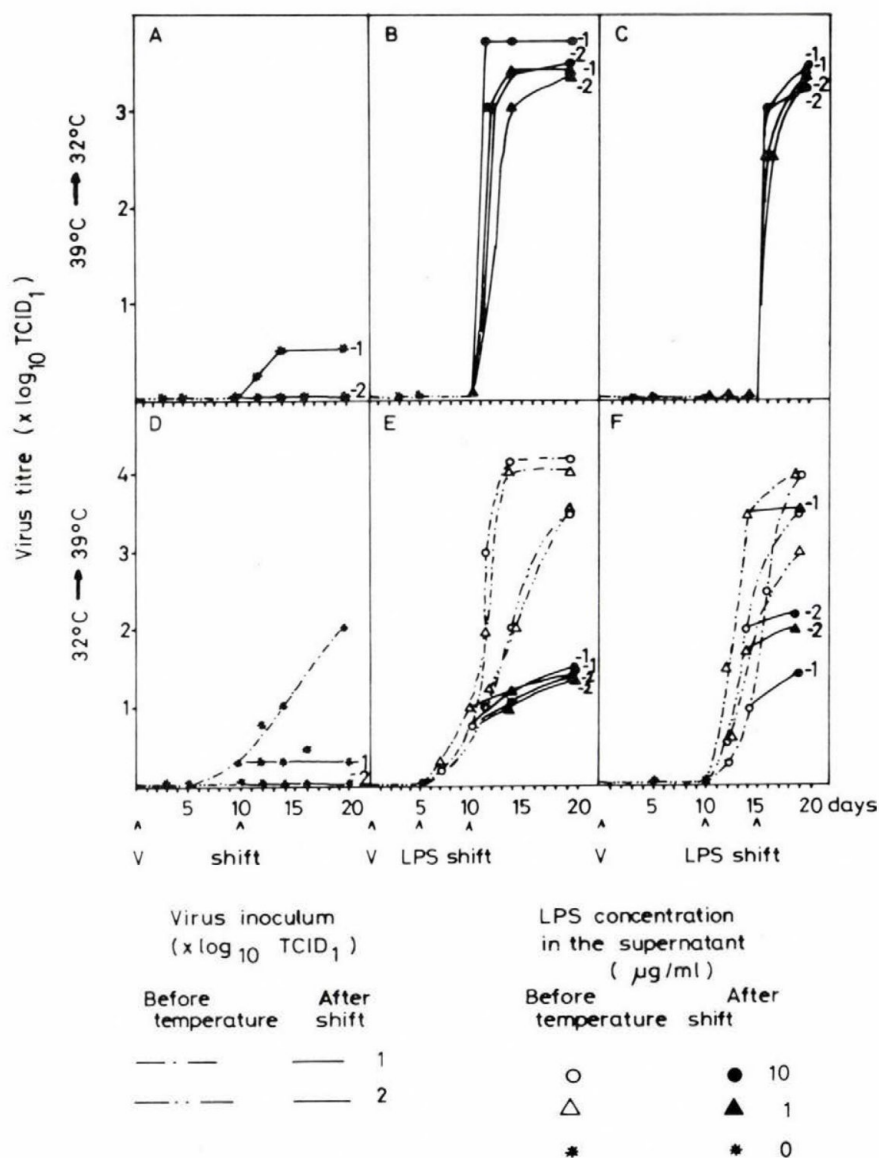


Fig. 3. Combined effects of LPS and temperature shift on Ad-5 mutant *ts18*

Effect of prednisolone and its combination with LPS on replication and reactivation. Prednisolone was not able to reactivate any type of latent adenovirus infection tested in this system, but it potentiated reactivation of latent infections by both LPS and RD-LPS at permissive temperatures and especially at 39 °C in the case of WT.

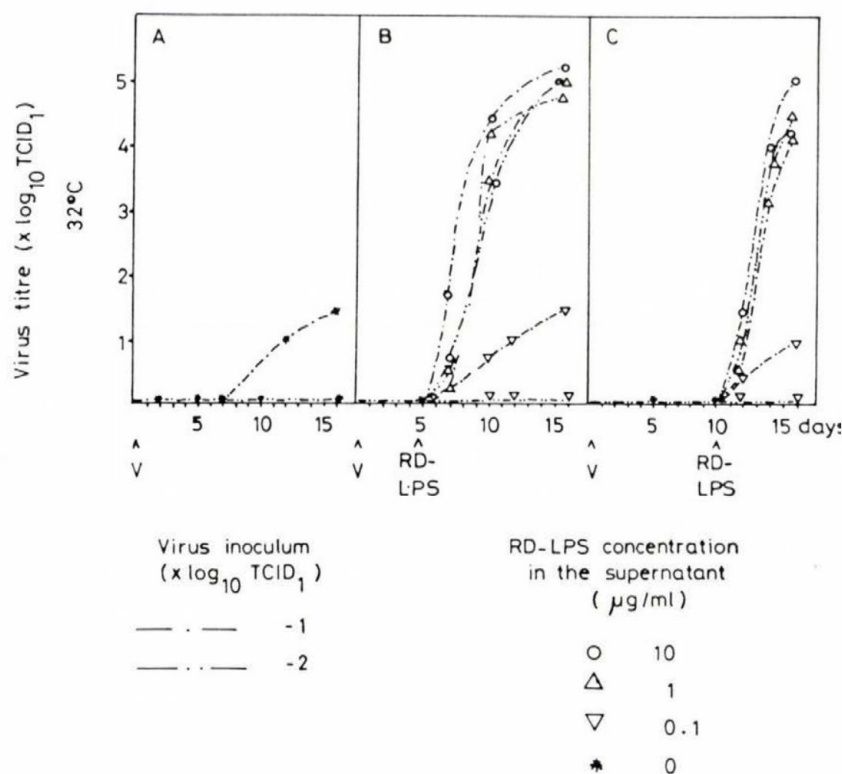


Fig. 4. The activating effect of RD-LPS on the replication of Ad-5 mutant *ts19*

Combined treatment with endotoxins plus prednisolone or late treatment at any time postinfection was able to induce limited but significant replication of mutant *ts19* at restrictive temperature. Shifting these activated cultures from 32 °C to 39 °C inhibited production of both *ts19* and *ts18* mutants only partially as compared to cultures, which were treated with either endotoxins or prednisolone alone (Table I). Preincubation of cells with prednisolone resulted in the increased slope of replication curves by viruses, which suggests that cells became more susceptible to adenovirus infections and/or their capacity to support virus replication became augmented. Prednisolone-enhanced adenovirus replication was hardly elevated by endotoxins, if these latter compounds were given later than 24 h from prednisolone application. In contrary, prednisolone given later, could further increase virus producing capacity elevated already by endotoxins, in spite of aging of cell cultures (data not shown). These mean that, prednisolone acts in synergism as a cofactor for endotoxins.

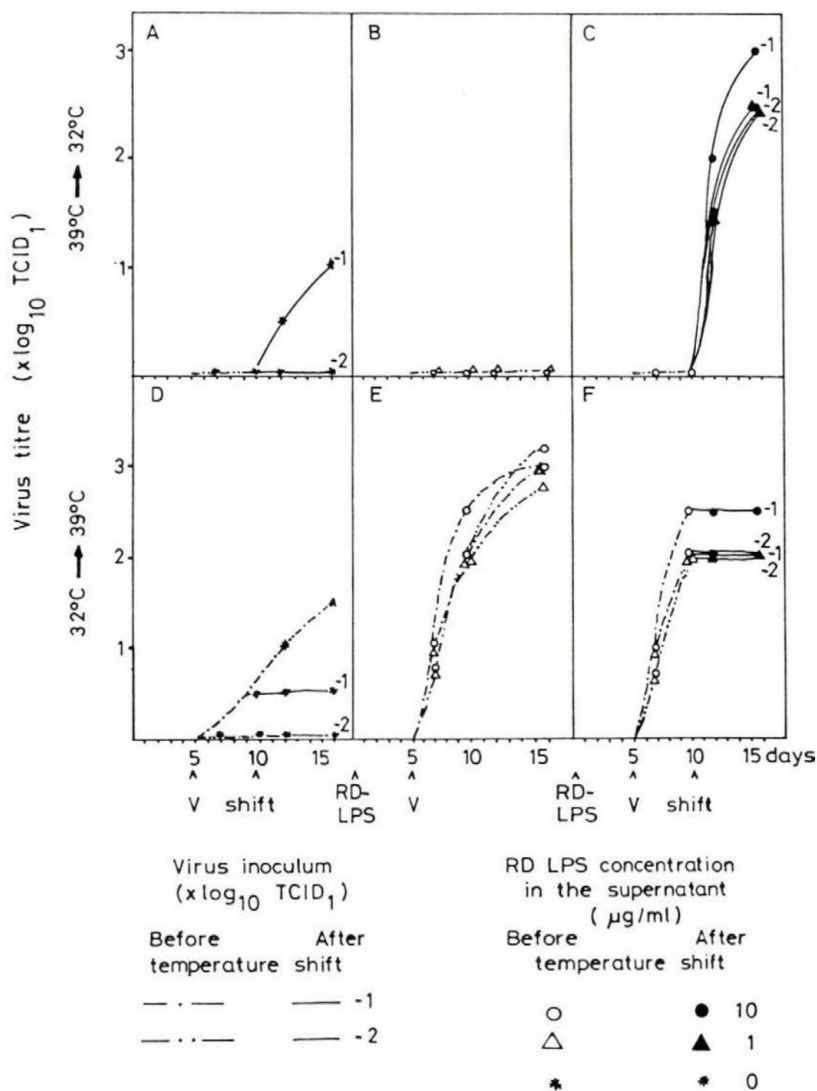


Fig. 5. Combined effects of RD-LPS and temperature shift on Ad-5 mutant *ts19*

Discussion

Reactivation of latent adenoviruses by endotoxins was a rapid and efficient process, was independent of the length of latency, and in certain range was independent of the concentration of latent virus, but depended on the concentration of LPS. These compounds were not able to increase virus producing capacity of cells

used, which suggests that, they act via cellular mechanisms without interfering viral gene expression. Radio-detoxification did not abolish activating ability of parental LPS, which could mean that, both types by binding to the same receptors act through membrane signalling [37, 44, 50] and cytoskeletal breakdown, which affects virus replication by reorganizing cell compartments [15, 24].

Another mechanism modifying Ad life cycle involves mediation through TNF- α [17]. Several non-immune cells produce TNF- α in response to LPS [38, 45–47]. Ad itself may induce TNF- α in both cell cultures [19] and mice [81], or alternatively TNF- α is produced by macrophages at the site of infection [19, 37, 39, 40, 73]. TNF- α might support a viral escape mechanism. Ad infected cells sensitized to lysis by TNF- α by expression of the E1A protein subsequently become resistant due to expressing the E1B 19 kD protein in human and the E3 14.7–10.4 kD and 14.5 kD proteins in murine cells [19]. E3 expression induced by TNF signal transduction may be mediated by NF- κ B [19, 81, 82]. Induction of E3 proteins by TNF- α has no direct effect on Ad replication, but rather influences interaction with its host [19]. In contrast, *high* doses of TNF- α and β inhibit replication of RNA- and DNA-viruses (e.g. Ad-2, HSV-2) [22, 81, 83].

Interaction of LPS with membranes results in deliberation of lysosomal enzymes [49, 50], which have a role in Ad uncoating [9]. Infecting lymphocytes with Ad-2, block in uncoating is found the major reason for nonpermissivity [18]. Uncoated particles are able to persist in cells for longer period both *in vitro* and *in vivo* [30]. In the recent experiments, both *ts* mutants and wild type Ad-5 were activated and enhanced in this way at 32 °C by LPS. α -Tocopherol known to counteract in membrane damages [51, 52], diminished these effects. Uncoating must be largely independent of special configuration of viral polypeptides, because preliminary experiments also showed activation and enhancement by LPS of Ad types 1, 8 and 12. It has been suggested that, viral proteinases may have a role in uncoating [84]. The adenovirus codes for a unique proteinase, which cleaves the pre-terminal protein (pTP), pVI, pVII, pVIII, IIIa, and a 11 kD protein, precursor of pX during virion maturation [3, 9, 20]. Although there is no evidence for cleavage in uncoating by this proteinase in the case of Ad, the Ad-2 *ts1* mutant, which lacks proteinase activity at 39 °C, is defective in uncoating. Its immature virions attach to cells but fail to yield productive infection [85]. Only a few copies of proteinase are present in each virion [9, 20], which may have a role in the processing of μ polypeptide, when the uncoated viral core is converted into a viral DNA-histone complex during a new infection process [9, 14]. Interestingly, the viral protease is able to cleave cellular cytokeratin during the late phase of infection [86]. Multiple cleavage sites have been demonstrated on cleaved proteins [3, 87]. These and our results suggest clear separation of the role of viral proteinase in maturation and not in viral uncoating in new infection. Mutant *ts18* is defective in phosphorylation of pVI, and mutant *ts19* is defective in phosphorylation of 11 kD polypeptide, therefore their abnormal precursors are not templates of viral proteinase at 39 °C [3, 14, 77]. Neither LPS could result in their replication at 39 °C, which means that, deliberated cytoplasmic enzymes cannot substitute adenovirus-encoded proteinase found in the nucleus [9].

Table I

The effects of endotoxins, α -tocopherol and prednisolone on Ad-5 WT and its mutants

Compound and concentration	Timing of treatment to infection	Event	WT		ts18				ts19			
			32 °C	39 °C	32 °C	32 °C → 39 °C	39 °C	39 °C → 32 °C	32 °C	32 °C → 39 °C	39 °C	39 °C → 32 °C
Native and detoxified endotoxins 10 ⁻¹ –10 ¹ µg/ml	Prior	Reactivation	+++	++	+++	0	0	++	+++	0	0	++
		Replication	+/-	0	+/-	---	0	++	+/-	---	0	++
	Late	Reactivation	+++	+	+++	0	0	++	+++	0	0	++
		Replication	-	0	-	--	0	++	-	---	0	++
α -tocopherol 10 ⁻¹ –10 ² µg/ml	Prior	Reactivation	0		0		0		0		0	
		Reactivation by endotoxins	--	ND	---	ND	0	ND	--	ND	0	ND
		Reactivation	--		--		0		--		0	
		Reactivation by endotoxins	---		---		0		---		0	
	Late	Reactivation	0		0		0		0		0	
		Reactivation by endotoxins	0	ND	0	ND	0	ND	0	ND	0	ND
		Reactivation	-		-		0		-		0	
		Reactivation by endotoxins	--		--		0		--		0	

Table I continued

21-prednisolone succinic acid sodium salt 4.0×10^{-7} M	Prior	Reactivation	0	0	0	0	0	0	0	0	0	0
		Reactivation by endotoxins	+++	+++	++	--	0	++	+++	--	+	++
		Reactivation	+++	+++	++	---	0	++	++	---	0	++
		Reactivation by endotoxins	+++	+++	+++	--	0	++	+++	--	+	+++
	Late	Reactivation	0	0	0	0	0	0	0	0	0	0
		Reactivation by endotoxins	+++	+++	+++	--	0	++	+++	--	+	++
		Reactivation	++	++	+	---	0	++	++	---	0	++
		Reactivation by endotoxins	+++	+++	+++	--	0	++	+++	--	+	+++

Quantitation:

- +++ = enhanced replication (2.6–3.5 lg)
 ++ = enhanced replication (1.6–2.5 lg)
 + = enhanced replication (0.6–1.5 lg)
 0 = no effect
 - = diminished replication (0.5–1.5 lg)
 -- = diminished replication (1.6–2.5 lg)
 --- = diminished replication (2.6–3.5 lg)
 ND = not done

Both mutants were able to infect and persist in cells at both permissive and nonpermissive temperatures, as temperature shift from nonpermissive to permissive also resulted in their reactivation. Activity of the adenovirus-encoded proteinase therefore contributes to regulating virus replication [89], and consequently it might be a possible target for chemotherapeutical inhibition without interfering cellular proteases [3].

Elevated PG levels are known to activate wide range of viruses [42], among them oncogenic and latent types of Ad [14]. In spite of possible elevated intracellular level of cGMP [37, 48] LPS was unable to activate any of these mutants [37, 48]. So, signal transduction after PG and after LPS treatments are different, and cellular PKs phosphorylating adenoviral polypeptides might require another cofactors [3].

Prednisolone did not activate any form of latent Ad infection [32], but it enhanced replication including that precedes reactivation. In humans, elevated virus replication exacerbating diseases are traced back to diminished number and function of immune, mainly T, cells, and suppression of IL-1, IL-2 production [51, 69]. But several in vitro experiments proved direct effect on viruses by steroid hormones [51]. Both the glucocorticoid and progesterone enhance MMTV expression and act in synergism with tyrosine kinase activity of the epidermal growth factor receptor (EGF-R) and lactogenic hormones. Transcription factors are implicated in their HRE activation [68, 73]. Cortisol in 10^{-7} M enhanced expression of HIV-1 LTR [68]. Glucocorticoids, when combined with various cytokines (TNF- α , IL-6, IFN- γ) can synergistically upregulate HIV expression in chronically infected promonocyte cells. Glucocorticoid and estrogen levels are high, but testosterone level is low in HIV infected persons. A similar mechanism occurs in septic or LPS shocks and stress situations. Concentration of arachidonic acid is high in the serum of patients with acquired immunodeficiency syndrome (AIDS) and AIDS related complex (ARC) [69]. Early in HIV infection, HPA response, including ACTH and cortisole, becomes abnormal and blunted to stress conditions [70], in which latter they are high, similarly to LPS effects [74]. Different pathways of LPS, other mitogens [39-41, 44, 88] and hormones [51] converge to influencing CNs and may act in synergism. Gp120 of HIV-1 inhibits both cAMP-enhancement by isoproterenol and production of TNF- α by LPS [89].

Among DNA viruses, HPV-16, -11, -18 contain distinct glucocorticoid/progesterone [59] and estrogen responsive elements [60]. Expression of E6 and E7 genes are enhanced by these hormones that leads to cellular immortalization [59, 60]. Glucocorticoid enhancement of HPV-16 plus activation of *ras* oncogene markedly enhance transformation of human keratinocytes [61] and baby rat kidney cells [63]. In the human cervix, metaplastic epithelium is the common target of steroid hormones including contraceptives and HPVs [62]. Biologically inactive hormone isomers are found without effect on polyoma virus replication, while anti-glucocorticoid effect of progesterone is enforced by decreasing activity of glucocorticoid in simultaneously treated cultures [64]. Reactivation of alpha-herpesviruses present in latently infected ganglia by dexamethasone in vivo resembles events of acute replication, but the effect is transient [58]. A proportion of human breast and gynecological cancers is known to be estrogen dependent. Cell lines (e.g. MCF-7) derived from them are tumourigenic in nude mice in the presence of estrogen, and their infection by herpes simplex virus type

1 or 2 (HSV-1, HSV-2) or transfection by virion protein Vmw65 gene stimulates expression of the estrogen receptor [57].

If a virus genome contains HREs, the effect of hormones is partially independent of intracellular processes. In contrast, interaction of Ads and steroid hormones seems to be more complex, and can affect several stages of Ad replication cycle. Estramurine-phosphate, an inhibitory estradiol-mustard conjugate interferes with the microtubule-mediated vectorial migration of Ad-2 inoculum to the nucleus of HeLa cells. Virus attachment and uncoating were not affected [90]. The course of Ad-5 infection in MCF-7 cells was increased by estradiol, diethylstilbestrol and dexamethasone [65, 91]. The increase of viral production was observed even if hormones had been present in the media until 6 h before infection. This mechanism clearly suggests involvement of cellular factors. Assembly proteins appeared earlier and production of particles with a higher infectious capacity during the early period of infection occurred. None of the steroids induced any modification of Ad-5 production in cells that lack steroid receptors [65]. Prednisolone accelerated Ad-5 replication by HEp-2 and primary human amnion cells, but diethylstilbestrol failed to do so [32]. These cells contain GR but might not be estrogen sensitive [91]. Transformation by Ad-12 in primary rat embryonic cells was enhanced by 17- β -estradiol, estrone, testosterone through enhanced viral adsorption that may suggest some interaction of steroid compounds with cell surface components. Transformed foci occurred earlier and in greater number in cultures of both female and male origin, and their oncogenicity became elevated in both rats and hamsters [66]. But transformation of primary hamster embryonic cells of both sexes by Ad-12 was enhanced by glucocorticoids, although slightly more foci developed in female cultures. Significant enhancement of transformation occurred in female cultures treated with cortison, and in male cells treated with dexamethasone. Marked inhibition of Ad-12 transformation occurred in both female and male cells treated with estrovarin, and of male cells treated with aldosterone or progesterone [67], which latter are known to be glucocorticoid antagonists in binding to cellular HRE [54, 55, 91]. Estrone and testosterone inhibited transformation of male cells slightly less markedly and had no effect on female cells. Although no details of pathomechanism could be concluded, it seemed unlikely that hormones acted directly on the virus [67]. E1A polypeptides cooperate with *c-jun* to activate transcription via AP-1 binding sites, that can confer enhanced E1A responsiveness to heterologous promoters [31]. The glucocorticoid hormone-receptor complex also utilizes *c-jun* homodimers bound at composite responsive elements when enhance transcription [54, 55]. Relative excess of free *c-fos* therefore might take part in transformation process [23, 31].

The moderate but significant replication of *ts19* mutant by the synergistic activation of prednisolone and LPS at 39 °C is the most intriguing question in this study. As the Ad-encoded proteinase cannot be substituted by cellular factors, partial restoration of damaged phosphorylation of the 11 kD protein is an explanation at this moment. Its simultaneous phosphorylation by PKs after augmentation of cGMP by LPS treatment [37] plus altered PK activity after the induction of AP-1 and another cellular transcription factors [2, 23, 31] might create such configuration of the precursor 11 kD polypeptide that, it becomes subject to proteolysis by the viral proteinase. All of these results draw attention to the importance of cellular factors in virus replication [3].

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CADMIUM MEDIATED CONTROL OF NITROGENASE ACTIVITY AND OTHER ENZYMES IN A NITROGEN FIXING CYANOBACTERIUM

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Cadmium decreased the specific growth rate and total cell mass at concentrations $>0.001 \mu\text{g ml}^{-1}$. It also reduced heterocyst frequency, total cell protein, nitrogenase activity (acetylene reduction) and decreased glutamine synthetase (GS) and glutamate synthase (GOGAT) activities. However, cells grown under low concentration of cadmium ($0.001 \mu\text{g ml}^{-1}$) had enhanced nitrogenase as well as GS activity besides showing increased growth rate and heterocyst frequency. Cadmium has apparently controlled nitrogenase activity and nitrogen fixation possibly via mediating enzymes including glutamine synthetase in *Nostoc muscorum*.

The exact mechanism of biological nitrogen fixation is being thoroughly investigated in several laboratories all over the world. The phototrophic systems including bacteria and cyanobacteria have emerged as important tools for research in this field [1–8]. Cyanobacteria have also been investigated in detail to envisage the effect of heavy metals on growth and basic nitrogen fixation [9–11]. However, the information on the effect of heavy metal mediated enzyme synthesis and regulation of nitrogen fixation is least understood in cyanobacteria. Since frequent presence of heavy metals detected in natural systems and habitats [12] and several cadmium-binding proteins have been isolated [13–14], it has become more important to investigate the interaction of cadmium with nitrogen fixation in order to establish its biological role. In this paper, we report the effect of cadmium on growth, differentiation of heterocysts, protein synthesis, nitrogenase synthesis and enzymes of nitrogen assimilation including glutamine synthetase and glutamate synthase. Biochemical control of nitrogen fixation has been analysed in relation to GS, GOGAT and nitrogenase in the background of cadmium.

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Materials and methods

Experimental material and growth conditions. *Nostoc muscorum*, a filamentous heterocyst-forming nitrogen fixing cyanobacterium has been used from our stock cultures as reported earlier [15]. Cyanobacterium was grown in modified BG-11 medium [16, 17]. After autoclaving and cooling the pH of the medium was 7.4. Phosphate and bicarbonate were autoclaved separately and mixed after cooling the medium to avoid precipitation. The cyanobacterium was grown in 100 ml conical (flat bottom) flasks fitted with cotton wool plugs. The cultures were maintained in an incubator at 27 ± 2 °C fitted with cool fluorescent illumination programmed for a 16:8 h light:dark cycle, with illumination approximately 1800 lux at the culture surface. The culture flasks were shaken at regular intervals to avoid clumping and sticking of the cyanobacterial cells to culture vessels and also to provide better aeration. Standard bacteriological techniques were used to maintain bacteria-free cultures. Growth was monitored in liquid cultures and specific growth rate constant K is described as $\log_{10}$ units day^{-1} selecting wavelength of 660 nm to ensure growth in terms of chlorophyll synthesis. Each point of absorbance on the growth curves represents a mean of three separate attempts.

Cadmium was supplied as its chloride. Double distilled and deionized water was used as a solvent. Metal solutions were freshly prepared in sterilized solvent to normalize a specified initial level of Cd^{2+} in the medium. Glassware was washed with 0.1 M HNO_3 before use to wash any adsorbed metal ion and then extensively rinsed with double distilled water.

Dry weight determinations. Aliquots were removed from culture and centrifuged for 20 min at 5000 g. The pellets were washed with distilled water and dried at 80 °C to constant weight.

Heterocyst frequency. The percentage of heterocysts was determined by counting a minimum of 1000 cells using ordinary light microscope. The results are expressed as the number of heterocysts present per 100 vegetative cells.

Estimation of total cell protein. Protein was estimated by (i) Lowry's method [18] and (ii) Sedmak and Grossberg method [19].

Nitrogenase activity (acetylene reduction). Nitrogenase was assayed by reduction of acetylene to ethylene [20, 21]. Nitrogenase activity was estimated after 72 h with initiation of log phase. The assay was done in stoppered 100 ml bottles containing 50 ml of cultures per cent volume of air (5 ml) was removed from the flasks and equal volume of acetylene was injected with a syringe through the rubber stopper. Incubations were carried out in a shaker bath at a photon fluence rate of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$. The reaction was terminated by injecting 1 ml of 20% trichloroacetic acid through the rubber stopper.

The amount of acetylene converted to ethylene was measured by NUCON 5700 gas liquid chromatograph. Gas samples (0.5 ml) from the sealed flasks were injected into the gas chromatograph equipped with hydrogen flame ionization detectors and a porapak N column. The operating conditions were as follows – oven temperature 105 °C detector/injector temperature 110 °C N_2 as carrier gas with a flow rate of 40 ml/min, H_2 as fuel gas with a flow rate of 20 ml/min.

Enzyme assays (GS and GOGAT). Crude cell-free extracts were prepared by sonication. Extracts used for all assays were prepared by centrifuging 5–10 ml suspension (exponential phase) and suspending the pellet in about 2.0 ml of ice-cold Tris-buffer (50 mM plus 2% mercaptoethanol, pH 7.8). Sonication was carried out at 4 °C applying 2–4 20s burst of energy at an estimated intensity of 100–110 mV. Cell debris was removed by centrifugation (refrigerated) for 30 min at 4 °C. The resulting supernatant was used directly for enzyme assays.

Glutamine synthetase (EC 6.3.1.2). GS was estimated using the transferase assay [22]. Assay mixture contained – 2 ml imidazole – HCl buffer (0.2 M); 4 ml glutamine (0.2 M); 0.6 ml MnCl_2 (0.1 M); 0.8 ml ADP (0.1 M); 0.4 ml potassium arsenate (1 M) and 2.2 ml glass distilled water; 0.5 ml of assay mixture was added to 0.2 ml of cell extract. The final volume was made up to 0.9 ml with 0.2 ml distilled water and to this 0.1 ml hydroxylamine solution was added. Incubations were carried out at 30 °C for 30 min. Reactions were terminated and colour developed by the addition of 2.0 ml of stop mixture (10% FeCl_3 , 24% trichloroacetic acid, 6 N HCl and distilled water mixed in the ratio of 8:2:1:13). Extinction was recorded at 540 nm in a Beckman spectrophotometer.

Glutamate synthase (EC 1.4.7.1). GOGAT was assayed by monitoring the oxidation of NADPH to NADP⁺ (indicated by a change in E_{340} nm [23]). Cell-free extract was same as used for glutamine synthetase. Assay mixture contained 0.05 ml cell extract, 0.1 ml tris buffer (0.5 M, pH 7.8) 0.05 ml α -ketoglutarate (0.1 M), 0.5 ml distilled water. The contents were mixed in a tube and absorbance was read at 340 nm in enzymeter (Calbiochem – Boehringer Corp), 0.05 ml of NADPH (5 mM) and 0.1 ml glutamine (0.1 M) were added to the above solution and absorbance recorded. Decrease in extinction was noted on per minute basis.

Suitable controls were incubated in all the assays to account for possible interference by reaction mixture components. Proper care was also taken to ensure that reaction velocities were linear with respect to the variables of protein concentration and elapsed time reaction.

Results

Growth curve of Nostoc muscorum in presence of different concentrations of cadmium is shown in Fig. 1. It is apparent that Cd²⁺ at low concentration (0.001 $\mu\text{g ml}^{-1}$) had stimulatory effect on growth while at higher concentration Cd²⁺ treated cells observed a gradual decrease in growth rate (Table I). Lag period was prolonged and continued up to 48 h in presence of Cd²⁺ (0.01 $\mu\text{g ml}^{-1}$). Further increase in Cd²⁺ concentration (0.10 $\mu\text{g ml}^{-1}$) proved to be lethal to the organism and completely inhibited growth followed by cell lysis and disruption.

Table I

Growth rate of N. muscorum in minimal medium (MM) and MM + cadmium (variable concentration)

Concentration of cadmium (mg ml ⁻¹)	Growth rate Doublings 24 h ⁻¹	Per cent change as compared to control
Control (BG 11)	0.34	—
0.001	0.37	2.9* (I)
0.01	0.29	14.7* (D)
0.10	0.22	35.3* (D)
1.0	Zero	100 (D)

I Increase

D Decrease

*. Significant

The decrease in cell mass varied from 3–35% depending on the age of culture and concentration of Cd²⁺ (Table II). Contrary to higher concentration, lower concentration of Cd²⁺ stimulated growth upto 3% of the cell populations selected through exponential phase. Clumping of cells was observed in response to high concentrations of Cd²⁺. Cd²⁺ at low concentration (0.001 $\mu\text{g ml}^{-1}$) increased heterocyst frequency by about 1.4%.

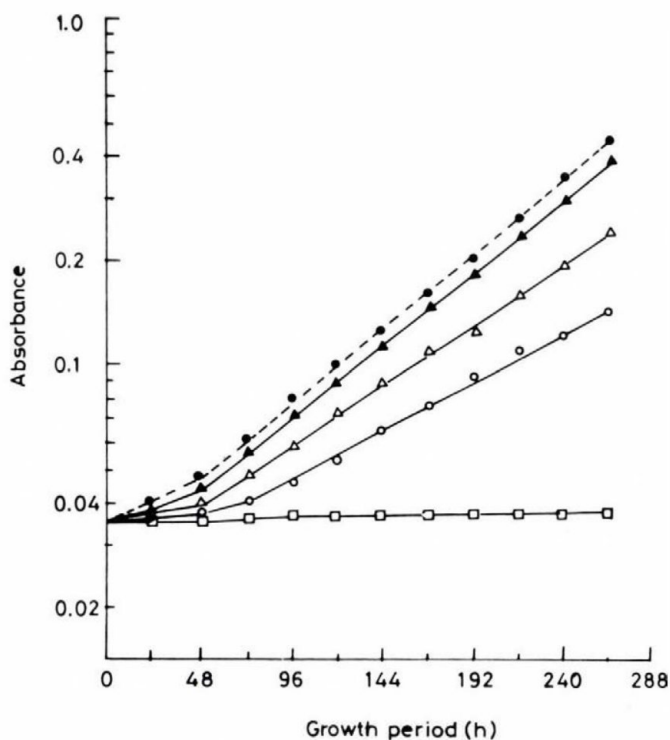


Fig. 1. Growth pattern of *N. muscorum* in minimal medium (MM) and MM + cadmium (variable concentration)

Table II

Dry weight of cells (mg 50 ml⁻¹) in MM + Cd²⁺

Age of culture (h)	MM	MM + Cd ²⁺ 0.001 µg ml ⁻¹	MM + Cd ²⁺ 0.01 µg ml ⁻¹	MM + Cd ²⁺ 0.10 µg ml ⁻¹
72	2.5	2.5	2.4	1.9
96	3.0	3.1	2.9	2.1
120	4.1	4.15	3.4	2.5
144	4.9	5.0	4.2	3.1
168	6.0	6.2	4.8	3.8
192	7.1	7.3	5.9	4.6
216	8.1	8.3	6.9	5.8
240	10.1	10.2	8.0	7.1
264	12.3	12.6	9.96	8.73

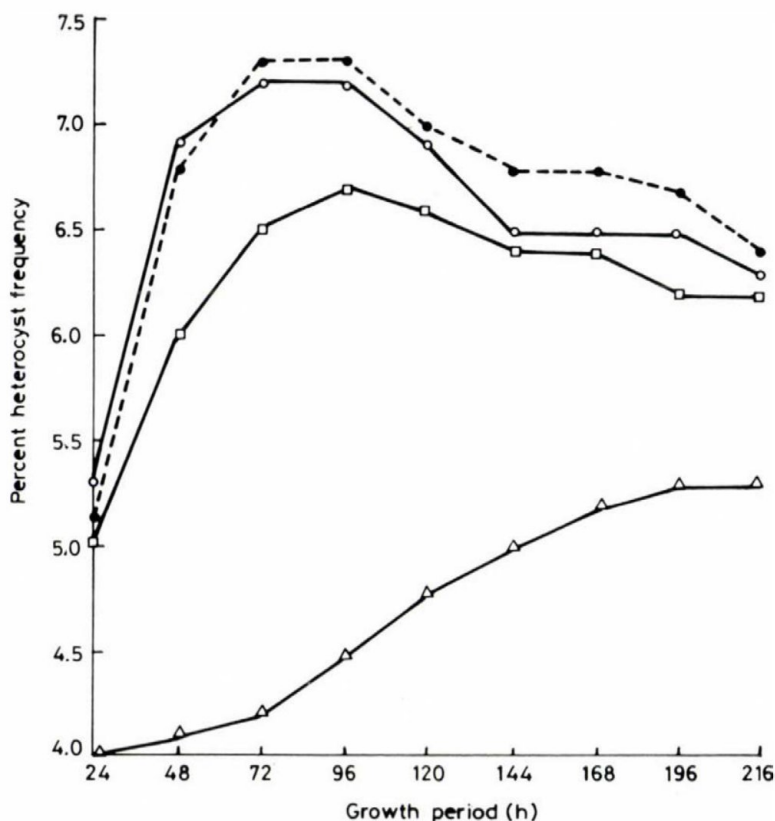


Fig. 2. Percent heterocyst frequency in *N. muscorum* grown in minimal medium (MM) and MM + cadmium (variable concentration)

At higher concentration, heterocyst frequency was decreased by 1 to 10% (Cd^{2+} $0.01 \mu\text{g ml}^{-1}$) and 14 to 41% (Cd^{2+} $0.10 \mu\text{g ml}^{-1}$) depending on the age of culture (Fig. 2). Effect of varying concentration of Cd^{2+} on total cell protein is shown in Table 3 selecting 216 h old culture on a comparative basis. Lower concentration of Cd^{2+} stimulated protein synthesis as estimated by both Lowry's and Sedmak and Grossberg methods. On the contrary, higher concentrations (0.01 to $0.10 \mu\text{g ml}^{-1}$) significantly decreased total cell protein.

Total nitrogenase activity (n moles C_2H_4 produced h^{-1}) and specific nitrogenase activity (n moles C_2H_4 produced $\text{h}^{-1} \text{mg dry wt}^{-1}$) in presence of different concentrations of Cd^{2+} is shown in Tables IV and V. Total nitrogenase activity as well as specific nitrogenase activity increased in MM supplemented with Cd^{2+} ($0.001 \mu\text{g ml}^{-1}$). Cells treated with high concentrations of Cd^{2+} (0.01 – $0.10 \mu\text{g ml}^{-1}$) exerted inhibitory effect on nitrogenase activity which decreased by 20.6 to 58% depending on

the age of culture. Specific nitrogenase activity also declined (ranging from 17 to 44%) in cells growing under higher concentrations of Cd^{2+} ($0.10 \mu\text{g ml}^{-1}$).

Table III

Total protein (age of culture 216 h) in BG 11 + Cd^{2+} estimated by Lowry's method (LM) and Sedmak and Grossberg's method (SG)

Concentration of cadmium ($\mu\text{g ml}^{-1}$)	Total cell protein ($\text{mg } 50 \text{ ml}^{-1}$)		Per cent change as compared to control	
	LM	SG	LM	SG
Control	2.31	2.88	—	—
0.001	2.38	2.99	3.0 (I)*	3.8 (I)**
0.01	1.82	2.24	21.2 (D)*	22.22 (D)**
0.10	1.40	1.72	39.39 (D)*	40.27 (D)**

I Increase

D Decrease

* Significant at 0.05 level

** Significant at 0.01 level

Table IV

Total nitrogenase activity (n moles C_2H_4 produced h^{-1}) in MM + Cd^{2+}

Age of culture (h)	MM	MM + Cd^{2+} $0.001 \mu\text{g ml}^{-1}$	MM + Cd^{2+} $0.01 \mu\text{g ml}^{-1}$	MM + Cd^{2+} $0.10 \mu\text{g ml}^{-1}$
72	58.85	58.90	46.75	24.70
96	70.09	72.5	56.98	31.47
120	84.21	86.7	64.6	38.75
144	98.21	102.39	77.00	52.80
168	118.02	125.20	87.80	63.5
192	138.04	148.30	98.90	73.60
216	156.88	163.15	111.90	90.63

Variation in GS and GOGAT activity was observed in cells treated with different concentrations of Cd^{2+} (Table VI). Lower concentrations of Cd^{2+} did not affect the activity of both the enzymes except for marginal change in GS activity (0.49%) in comparison to untreated cells. Cells treated with higher concentrations of Cd^{2+} ($>0.001 \mu\text{g ml}^{-1}$) observed decline in GS and GOGAT activity as indicated.

Table V

Specific nitrogenase activity (n moles C₂H₄ produced h⁻¹ mg dry wt⁻¹) in MM + Cd²⁺

Age of culture (h)	MM	MM + Cd ²⁺ 0.001 µg ml ⁻¹	MM + Cd ²⁺ 0.01 µg ml ⁻¹	MM + Cd ²⁺ 0.10 µg ml ⁻¹
72	23.54	23.86	19.40	13.00
96	23.36	23.74	19.65	14.99
120	20.53	20.89	19.00	15.50
144	20.45	20.69	18.80	16.00
168	19.67	19.75	18.30	16.30
192	19.44	19.70	17.99	16.00
216	19.36	19.65	17.20	15.90

Table V

Specific glutamine synthetase (GS) and glutamate synthase (GOGAT) activity in MM + Cd²⁺ (age of culture 216 h)

Concentration of cadmium (µg ml ⁻¹)	GS	GOGAT	Per cent change in activity as compared	
	(µ moles min ⁻¹ mg protein ⁻¹)	(µ moles min ⁻¹ mg protein ⁻¹)	GS	GOGAT
Control	1.03	0.002	—	—
0.001	1.035	0.002	0.49 (I)*	Nil
0.01	0.841	0.0018	18.3 (D)*	10 (D)*
0.10	0.695	0.0011	32.5 (D)*	45 (D)*

I Increase

D Decrease

* Significant at 0.05 level

Discussion

The increase in growth in presence of low concentrations of cadmium (0.001 µg ml⁻¹) was very well reflected in the increased total cell mass, increased total nitrogen (data not shown) and increased total cell protein. Concomitantly, there was an increase in total nitrogenase activity as well as specific nitrogenase activity in presence of low concentration of cadmium. Cadmium, therefore, at low concentration, stimulated all the metabolic processes studied. However, there was not any change observed in GOGAT activity at this concentration in contrast to GS which increased. The stimulation of both GS and nitrogenase activity, barring GOGAT, further supports the role of GS as a modulator for nitrogen fixation besides other evidences as already

propounded by other workers [24–26, 15]. High concentrations of cadmium ($0.001 \mu\text{g ml}^{-1}$) resulted in decrease in overall metabolism as reflected in all the above noted processes and activities. Cadmium is already known to be toxic in several systems including bacteria [27, 28], cyanobacteria [29, 30], green algae [31, 32, 33] and higher plants [34–36]. However, the mechanism of stimulation and inhibition of metabolic processes by cadmium have not been worked out in detail. Some cadmium-binding proteins have been isolated from plants [14, 37]. It has been reported that microbial protein forms a water insoluble precipitate with cadmium which plays a major role in decreasing the concentration of soluble cadmium during microbial growth [41]. It has also been observed that cadmium affects metallothioneins which are low molecular weight proteins unusually rich in cysteine content and have important biological role [38–40]. Metallothionein type of proteins which bind to cadmium have also been isolated in cyanobacteria [13]. Therefore, it is imperative that cadmium might be controlling the cell metabolism at low concentrations through metallothioneins and its stimulatory and toxic effects could easily be envisaged. In the absence of suitable mutants, it is very difficult to propound a theory for cadmium binding regulatory protein concerned with nitrogen fixation. It has been reported that nitrogenase activity was suppressed by 48 h under Cd stress in cyanobacteria [42]. However, reports correlating nitrogenase activity with GS and GOGAT are not given. Apparently glutamine synthetase and nitrogenase is one such example which, in the present investigation has a clearly indicated to be governed by cadmium and possibly via mediating proteins through repression and derepression mechanisms.

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SOLUBLE INTERCELLULAR ADHESION MOLECULE-1 (sICAM-1) IN GRAVES' DISEASE

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The aim of this study was to determine the level of sICAM-1 in sera of hyperthyroid and euthyroid patients with Graves' disease and find correlation between thyroid function, anti-TSH receptor as well as anti-thyroid antibodies. Comparative determination of sICAM-1, TSH and thyroid hormones, anti-TSH receptor – as well as anti-thyroglobulin –, anti-thyroid peroxidase antibodies was made in sera of Graves' patients. Thirty patients with untreated hyperthyroid Graves' disease (12 had infiltrative ophthalmopathy) were studied before and after methimazole treatment. sICAM-1 was measured by ELISA using monoclonal antibodies to human ICAM-1. The sICAM-1 was significantly elevated in hyperthyroid Graves' patients (738.9 ± 151 ng/ml) compared to controls (212 ± 42 ng/ml) ($p < 0.0001$). Both in hyperthyroid and euthyroid Graves' groups with infiltrative ophthalmopathy the sICAM-1 proved to be higher than those without ophthalmopathy. Correlations were observed between sICAM-1 and serum thyroxine, triiodothyronine and anti-TSH receptor antibodies ($r = 0.91, 0.83$ and 0.61 , respectively). There was, however, no association of serum ICAM-1 levels with anti-thyroid peroxidase or anti-thyroglobulin antibodies. It was concluded that elevation of sICAM-1 can be the consequence of hyperthyroidism and the autoimmune processes mediated by anti-TSH receptor antibodies as well as cytokines. The elevated level of sICAM-1 might be important both in regulation of autoimmune mechanism and "homing" phenomenon of lymphocytes in Graves' disease.

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin supergene family and functions as a ligand for the lymphocyte function-associated antigen-1 (LFA-1) [1, 2]. Normal and pathological distribution of ICAM-1 has not yet clarified. It is known that ICAM-1 can be expressed on endothelial, thymic epithelial cells, macrophages and dendritic cells [2]. Increased expression of ICAM-1 has been observed on human thyroid cells obtained from the patients with autoimmune thyroid diseases [3, 4]. Furthermore, ICAM-1 expression by cultured human thyrocytes was enhanced with cytokines including TNF, IFN gamma and IL-1. The interaction of ICAM-1 with LFA-1 is an initial, essential step in the immune response and seems to be important in perpetuation of the local autoimmune response in autoimmune thyroid diseases [5]. Structurally, ICAM-1 consists of five extracellular immunoglobulin-like domains, a single transmembrane region and a short

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cytoplasmic tail [6, 7]. The extracellular part of ICAM-1 molecule can be shed from cells induced by cytokines [8, 9]. Shedding of ICAM-1 of in a target organ including thyroid gland in autoimmune thyroid disease might result in the appearance of soluble ICAM-1 (sICAM-1). The aim of this study was to test this possibility and test whether thyroid hormone level can influence the sICAM-1.

Materials and method

Patients and control group. Thirty patients (28 female, 2 male) aged 22–62 years, average 41.4 years with untreated hyperthyroid Graves' disease were studied. The diagnosis of thyrotoxicosis was based on standard clinical criteria and laboratory findings. All patients were hyperthyroid and 12 had infiltrative ophthalmopathy according to the criteria of the American Thyroid Association. Thyrotoxicosis was confirmed by high level of triiodothyronine (5.96 ± 2.7 nmol/l, normal range 1.5–3.0 nmol/l) and thyroxine (224 ± 38 nmol/l, normal range 65–160 nmol/l) measured by Amerlite TT₃ and TT₄ assays (Amersham, Bucks, UK). TSH of patients' sera was suppressed: <0.15 mU/l, normal range 0.15–3.2 mU/l (Amelitte TSH assay, Amersham). The hormones were detected in euthyroid patients induced by Methimazole therapy (TT₃ and TT₄ were 1.87 ± 0.4 nmol/l, and 121 ± 23 nmol/l, TSH 2.1 ± 0.42 mU/l, respectively). The age- and sex-matched control group consisted of 20 healthy subjects (18 female, 2 male, 20–52 years of age, average 42.1 years).

Anti-TSH receptor antibodies were detected by radioreceptor assay (TRAK, Henning Chemie und Pharmawerk, Berlin) and expressed in U/l. Values above 12 U/l were considered positive.

Anti-thyroglobulin antibodies were detected by ELISA published elsewhere [12]. Results were considered to be positive above 200 (mean $\pm$ 2SD).

Anti-peroxidase antibodies were measured by radioimmuno-assay using monoclonal antibody (Dynotest anti-TPO, Henning Chemie und Pharmawerk, Berlin) and were positive above 50 U/l.

Determination of sICAM-1 was carried out in sera by enzyme immunoassay using monoclonal antibody to human sICAM-1 (Bender MedSystems, Austria) with standard method. Briefly, anti-sICAM-1 monoclonal antibody was adsorbed onto polystyrene wells. sICAM-1 present in the diluted (1:100) sera and standard was bound to antibodies to wells. After washing with PBS (pH 7.4) a horseradish peroxidase conjugated monoclonal antibody with function neutralizing properties against sICAM-1 was added and bound to the sICAM-1 captured by the first antibody. Unbound enzyme conjugated anti-sICAM-1 was removed during a wash step and the substrate (tetramethyl-benzidine) solution reactive to horseradish peroxidase was added to the wells. A coloured product was formed in proportion to the amount of sICAM-1 molecule and measured Dynatech ELISA reader (450 nm). Twenty healthy aged and sex matched sera of blood donors were tested and sICAM-1 levels were between 155–356 ng/ml.

Statistical analysis. The results were expressed as mean $\pm$ SD. The data were analysed with a Student's *t* test for unpaired samples and linear regression, multiple regression and analysis variance (ANOVA, PS-Plot, Salt Lake City, USA) to examine the relationship between sICAM-1 and thyroid hormones and other laboratory parameters.

Results

Study of sICAM-1. We have found a significant increase of sICAM-1 in sera of patients with untreated Graves' disease (738.9 ± 151 ng/ml) compared to controls (212 ± 42 ng/ml) ($p < 0.0001$). Interestingly, sICAM-1 level was higher in patients with infiltrative ophthalmopathy (828 ± 153 ng/ml) than in those without eye symptoms (659 ± 102 ng/ml) (Fig. 1.). In euthyroid Graves' patients the mean value of sICAM-1

remained higher (267 ± 53 ng/ml) than in controls, however, this difference was not significant. Furthermore, in euthyroid patients with infiltrative ophthalmopathy the sICAM-1 was significantly enhanced (325 ± 58 ng/ml) compared to those without ophthalmopathy (228 ± 49 ng/ml) ($p < 0.05$).

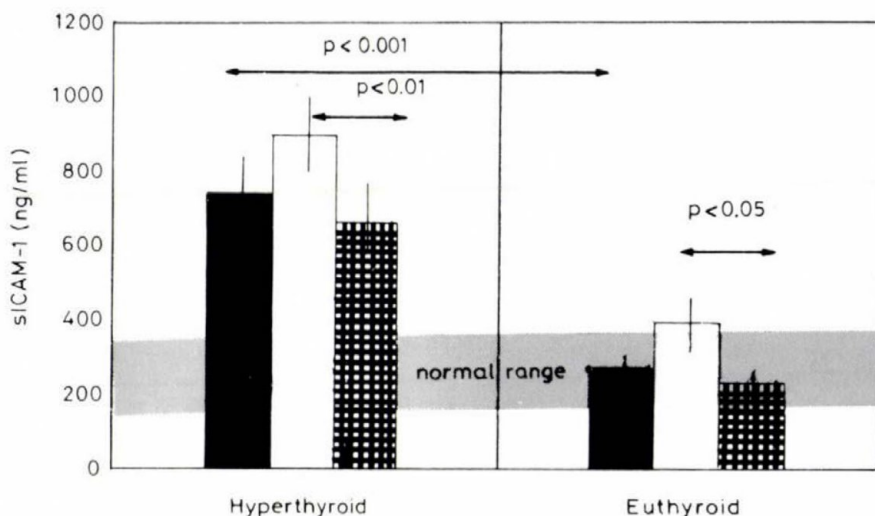


Fig. 1. Investigation of sICAM-1 in hyper- and euthyroid Graves' patients. The solid columns show all the patients, open and crossed columns separately demonstrate the patients with and without ophthalmopathy.

Correlation between sICAM-1 and other laboratory parameters. A correlation was observed between sICAM-1 and serum triiodothyronine and thyroxine levels ($r=0.83$ and $r=0.91$, respectively) (Fig. 2). The correlation coefficient was lower between sICAM-1 and anti-TSH-receptor antibodies ($r=0.61$) (Fig. 3). In contrast, there was no association between anti-thyroid peroxidase as well as anti-thyroglobulin antibodies.

Discussion

It is known that cytokine(s) and Graves' immunoglobulins with high level of anti-TSH receptor antibodies are able to enhance the ICAM-1 as well as HLA class II on the surface of thyrocytes and orbital fibroblasts [5, 15, 16]. As demonstrated in recent studies, coexpression of these molecules is critical for cell contact, antigen presentation, HLA class II antigen restricted cell specific T cell activation and perpetuation of autoimmune mechanisms [4]. The ICAM-1 molecule has been known to belong to "homing" receptors of thyroid which may play a decisive role in the early phase of organ specific autoimmunity [10]. The possible source of sICAM-1 is Graves' thyroid glands, endothelial cells and orbital fibroblasts however, the precise mechanism of shedding of this molecule has not yet been clarified.

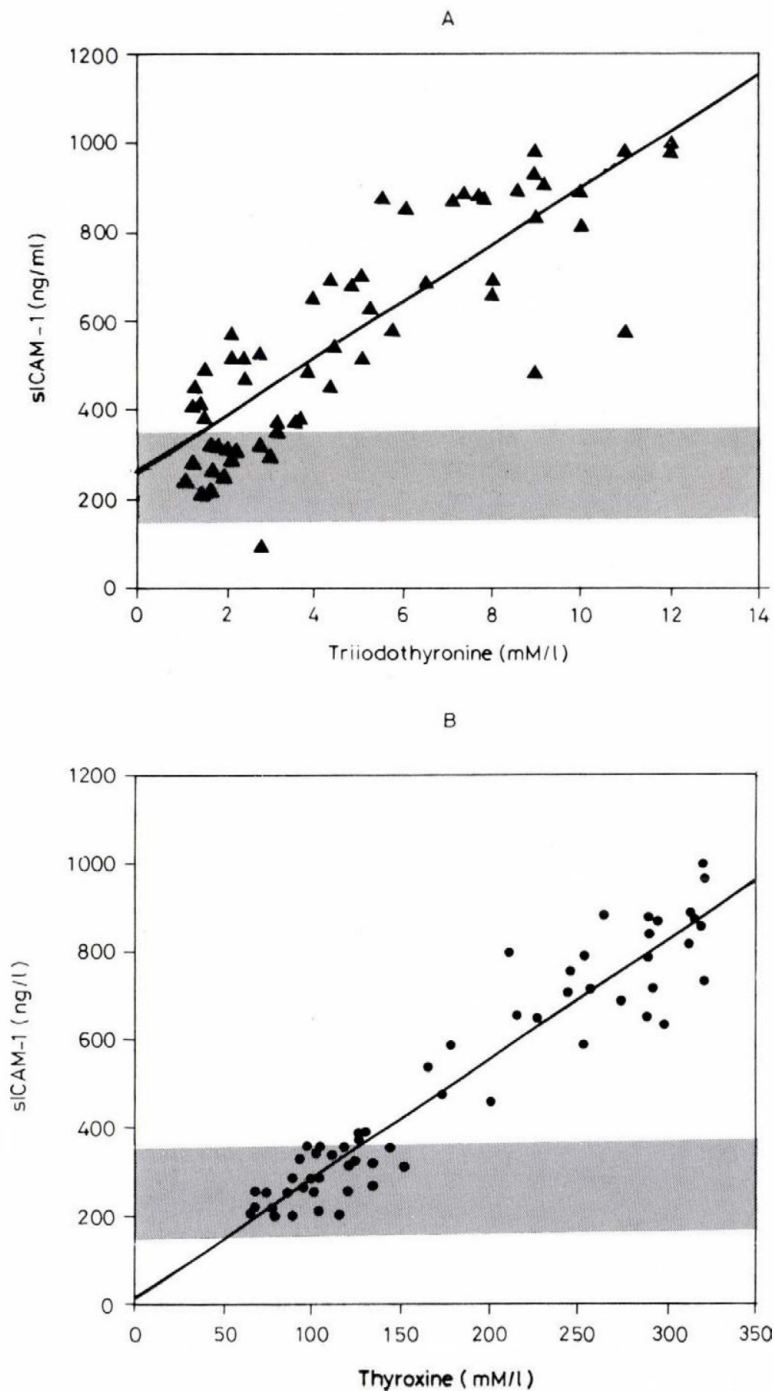


Fig. 2. Correlation between sICAM-1 and triiodothyronine (A) and thyroxine (B) in Graves' pat. Correlation was found with thyroxine and triiodothyronine ($r=0.91$ and 0.83 , respectively)

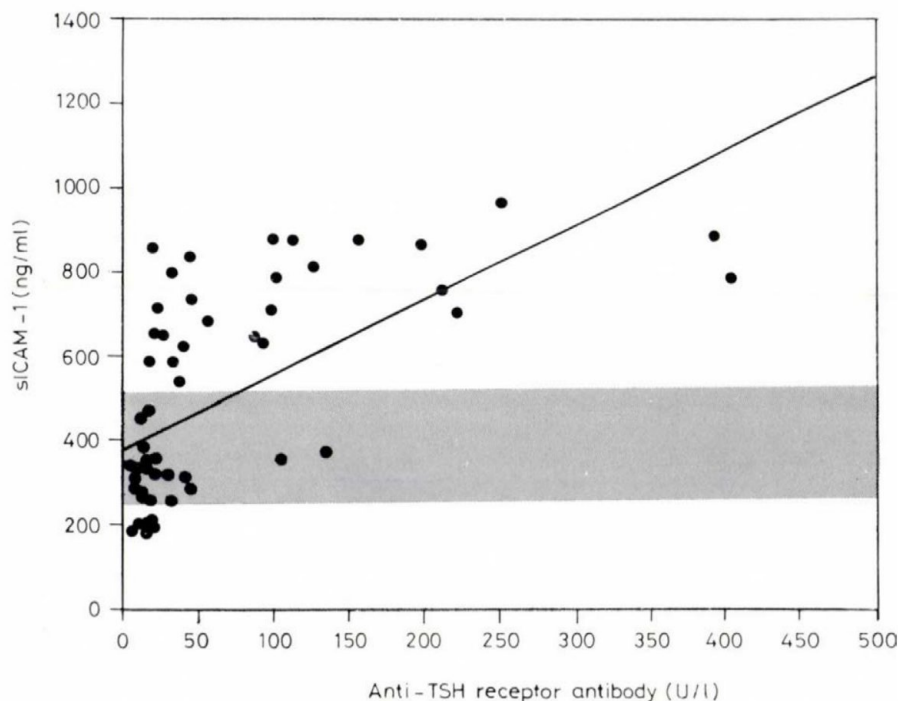


Fig. 3. Correlation between sICAM-1 and anti-TSH receptor antibodies ($r=0.61$)

Our observation on higher level of sICAM-1 in patients with ophthalmopathy irrespectively of thyroid function suggest that the orbital fibroblasts can shed sICAM-1. Nevertheless sICAM-1 is unlikely to be a disease-specific marker since the increased sICAM-1 has been found in sera of patients with leukocyte adhesion deficiency (LAD), neoplastic disorders as well as allograft rejection [7-9]. sICAM-1 could be theoretically the product of proteolytic cleavage of cell-bound ICAM-1 close to the cell membrane. Alternatively, sICAM-1 might be the product of an alternatively spliced messenger RNA which effectively deletes the transmembrane and cytoplasmic domains. The later possibility is unlikely since to date there is no experimental evidence of an alternatively spliced form of ICAM-1 mRNA that would lead to the expression of sICAM-1. We have found the fairly correlation between sICAM-1 and thyroid hormone levels. In spite of this observation we suppose that the thyroid hormone might not be responsible for increased shedding of sICAM-1. Furthermore, both enhanced levels of sICAM-1 and thyroxine, triiodothyronine appear to be the consequence of Graves' immunoglobulin and cytokine induced perturbation of thyroid membrane.

The role of soluble receptors including sICAM-1, soluble IL-2 receptor in immune regulation is the matter of studies [9, 11]. Conceivably, sICAM-1 could compete with membrane ICAM-1 on LFA-1 positive lymphocytes. LFA-1 can be

blocked with sICAM-1, therefore, it seems likely that enhanced level of sICAM-1 is responsible, at least in part, for the decreased number of circulating LFA-1 positive lymphocytes in Graves' disease [11]. Drexhage et al. [14] have demonstrated the existence of a decreased attachment of Graves' lymphocytes to high endothelial venules (HEV). Since LFA-1 is involved in HEV binding via the ICAM-1 molecules, diminution of LFA-1 positive lymphocytes by binding sICAM-1 could explain the decreased attachment to HEV and the less important lymphocytic infiltration in Graves' glands compared to Hashimoto's thyroiditis [4].

We speculate that the overproduction of cytokines by autoreactive T cells and anti-TSH receptor stimulating antibodies are responsible for the strong expression of ICAM-1 on thyrocytes. Shedding ICAM-1 may block the attachment of autoreactive cells ("homing"), therefore, it might be one of the mechanisms via the thyroid cells can escape the autoreactive T cells. Although several experimental data support this hypothesis concerning malignant disease, however, in autoimmune disorders this suggestion requires further elucidation.

Acknowledgements. This work was supported by Ministry of Welfare, Hungary (Grant T-539/1990).

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THE EFFECT OF X-RADIATION ON RETICULO- ENDOTHELIAL SYSTEM AND ITS TREATMENT WITH RADIODETOXIFIED-ENDOTOXIN AND TRACE ELEMENTS IN RATS

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A new *in vivo* method has been developed for the precise observation of RES activity. Both *Escherichia coli* endotoxin 100 µg/100 g i.v. (LPS) and radiodetoxified endotoxin 100 µg/100 g body weight i.v. (RD-LPS, TOLERIN) both increased the granulopoietic activity of RES. The RD-LPS was more effective. The preparation containing trace elements also increased the activity of RES. The treatment consisting of the use of both trace elements and RD-LPS proved to be the most effective. The activity of RES was inversely proportional to various doses of X-ray irradiation (7, 8, 9 Gy). Trace elements and RD-LPS even improved the immunity system of animals having deteriorated RES.

Phagocytosis by cells of reticuloendothelial system (RES) is an essential defense mechanism against infection. The fixed macrophages lining the vascular compartment constitute an important group of cells of the RES. The majority of these macrophages are located in the sinusoids of the liver and spleen. Activity of these cells can be investigated with "clearance-tests" when collides are administered intravenously and the rate of clearance of particles from the blood is measured [1]. The effect of X-ray and ethanol consumption on the activity of RES with clearance-test has been described in the literature. Early studies [2] demonstrated the importance of the RES in prevention of radiation-induced bacteraemia. Benacerraf et al. [3] reported that exposure of rats to 850 R of X-irradiation inhibited phagocytic recovery from carbon-induced phagocytic blockade. Di Luzio [4] observed that the rate of clearance of a tracer dose of colloidal gold was not affected by radiation, although tissue distribution of the collide was altered. Saba et al. [5] demonstrated that whole body X-irradiation with 800 R markedly impaired the phagocytosis of a gelatinized ¹³¹I-labelled triolein in adult rats. But other studies reported that irradiation stimulates the RES [6, 7] or has no effect at all [8, 9].

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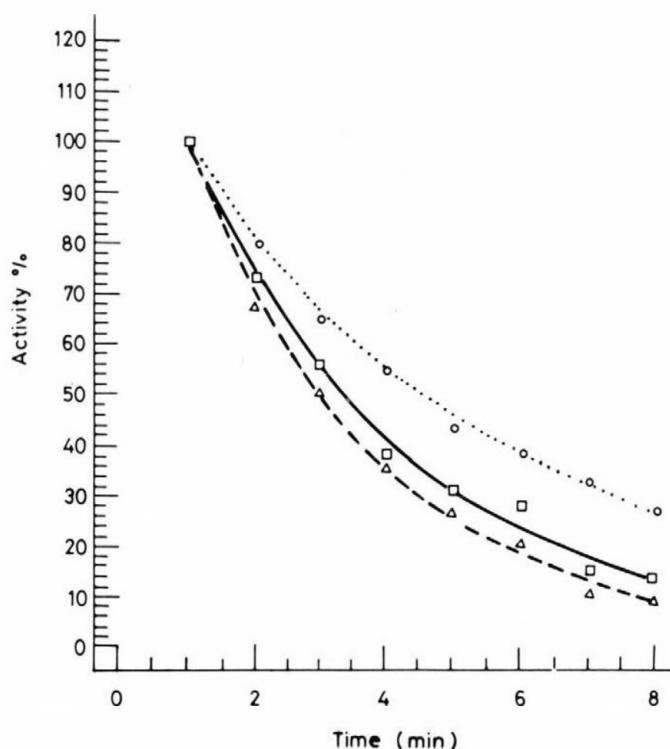


Fig. 1. The effect of endotoxin (LPS) on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. Endotoxin was injected i.v. 24 h before the RES study. □ K_{control} : 0.2928 ± 0.0122 $T_{1/2}$: 2.37 min; △ $K_{\text{LPS } 100 \mu\text{g}}$: 0.3371 ± 0.0124 $T_{1/2}$: 2.05 min; ○ $K_{\text{LPS } 200 \mu\text{g}}$: 0.1883 ± 0.0078 $T_{1/2}$: 3.68 min

In the present study we observed the effect of various materials and interventions on RES in vivo. We used radiodetoxified endotoxin (RD-LPS) [10–12] and/or a special preparation containing trace elements [13] to moderate these damaging effects of whole body X irradiation on RES of rats. There are no publications about the effects of combination of these interventions and treatments on the reticulo-endothelial system.

Materials and methods

Animals. We used 170 female Wistar rats from LATI (Gödöllő) weighting 140–150 g. They were fed with granulated chow (LATI, Gödöllő) and tap water ad libitum. Animals used this study were maintained and used in accordance with the "Guide for the Care and Use of Laboratory Animals" (Hungarian Academy of Sciences).

Preparation of microaggregated nanoalbumin. The microaggregated ^{99m}Tc labelled nanoalbumin (NANOALBUMON) was prepared in "Frederic Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene. The particle size was 50–80 nm.

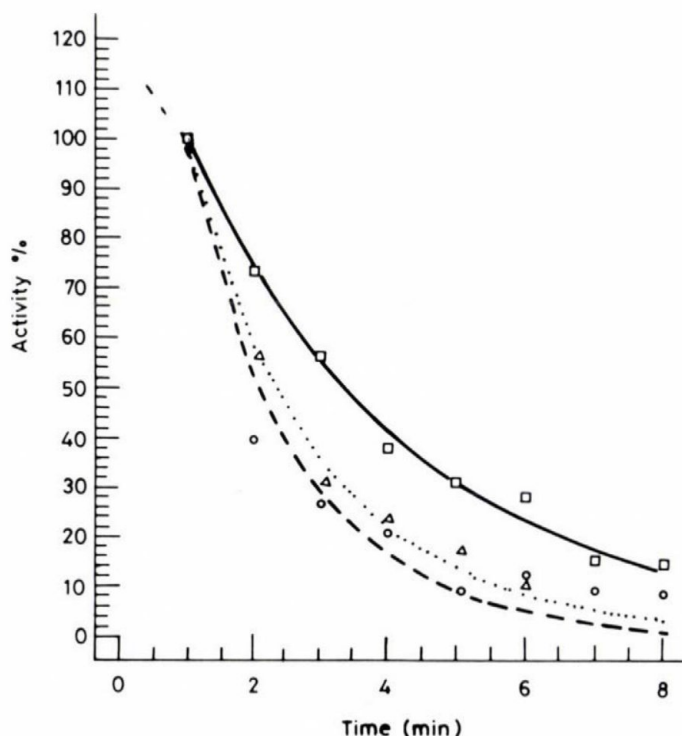


Fig. 2. The effect of radiodetoxified endotoxin (RD-LPS) on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. RD-LPS was injected i.v. 24 hours before the RES study. $\square$ K_{control} : 0.2928 ± 0.0122 $T_{1/2}$: 2.37 min; Δ $K_{\text{RD-LPS } 100 \mu\text{g}}$: 0.4928 ± 0.0495 $T_{1/2}$: 1.41 min; $\circ$ $K_{\text{RD-LPS } 200 \mu\text{g}}$: 0.5749 ± 0.0924 $T_{1/2}$: 1.20 min

Preparation of endotoxin. The *E. coli* endotoxin (LPS) was prepared in our laboratory according to a modified method of Westphal et al. [14].

Preparation of radiodetoxified endotoxin. The radiodetoxified endotoxin (RD-LPS, TOLERIN) was prepared in our laboratory from *E. coli* endotoxin irradiated with 150 kGy ^{60}Co gamma-ray [15].

Trace element treatment. A special trace element preparation (Béres Drops Plus; Béres Inc., Hungary) was given to 4 groups of animals through a probe at doses of 28 drops (2 ml) every day for 4 weeks. The trace element preparation contains Fe, Zn, Na, Mg, K, Cu, Mo, V, Ni, B, F, Cl, Co, glycerol, EDTA, L-(+)-tartaric acid, succinic acid, L-(+)-ascorbic acid.

Radiation. The whole body irradiation of the animals was made with a THX-251 X-ray device at 200 kV, 20 mA, 60 cm focus-body-middle distance, 0.5 mm Cu screening. Dose output, 0.498 Gy/min. The groups received 7, 8 or 9 Gy X-ray doses.

Experimental groups. 1.1. Control. 1.2. 100 µg LPS injected i.v. 24 h before the RES activity study. 1.3. 200 µg LPS injected i.v. 24 h before the RES activity study. 1.4. 100 µg RD-LPS injected i.v. 24 h before the RES activity study. 1.5. 200 µg RD-LPS injected i.v. 24 h before the RES activity study.

2.1. Control. 2.2. Trace element treatment for 4 weeks before the experiment. 2.3. 100 µg RD-LPS injected i.v. 24 h before the RES activity study. 2.4. Combination of trace element and RD-LPS treatment

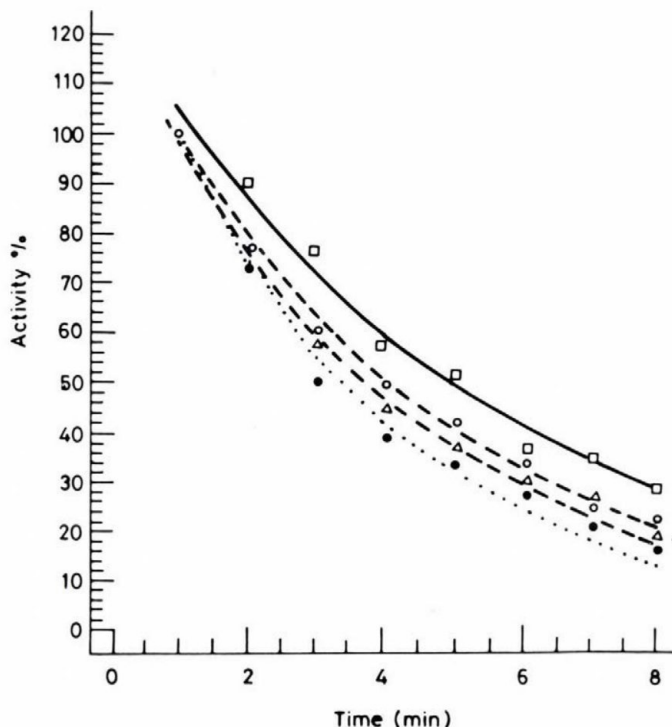


Fig. 3. The effect of RD-LPS and trace elements on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. □ K_{control} : 0.1874 ± 0.0109 $T_{1/2}$: 3.70 min; ○ $K_{\text{RD-LPS } 100 \mu\text{g}}$: 0.2223 ± 0.0072 $T_{1/2}$: 3.12 min; Δ $K_{\text{trace elements}}$: 0.2312 ± 0.0098 $T_{1/2}$: 3.00 min; ● $K_{\text{RD-LPS} + \text{trace elements}}$: 0.2670 ± 0.0159 $T_{1/2}$: 2.59 min

3.1. Control. 3.2. 9 Gy X-ray 4 days before the RES activity study. 3.3. 8 Gy X-ray 4 days before the RES activity study. 3.4. 7 Gy X-ray 4 days before the RES activity study.

4.1. 8 Gy X-ray 4 days before the RES activity study. 4.2. 8 Gy X-ray 4 days, 100 µg RD-LPS 1 day before the RES study. 4.3. Trace element treatment for 4 weeks and 8 Gy X-ray 4 days before the RES study. 4.4. Combination of trace element and RD-LPS treatment with e Gy X-ray.

Experimental method. Animals were anaesthetized by Nembutal (0.004 g/100 g body weight). Both v. jugularis were prepared, a canule (1 mm I.D.) was led into one of the veins. The 50 µg ^{99m}Tc labelled nanoalbumin microcolloid was injected in the other vein. Eight blood samples were taken through the canule at 1 min intervals.

Calculation of results. The weight and the radioactivity of the blood samples were measured and a calculation was made for 1 g of blood. Regarding the first blood sample as 100% the other samples were compared to this. Data were expressed in diagrams and exponential curves were fitted by STATGRAPHIC computer software. We compared the different Granulopoietic Indices (K) [1]. The difference between these indices was subjected to a standard *t*-test ($p=0.05$, $T_{1/2}$ = half time).

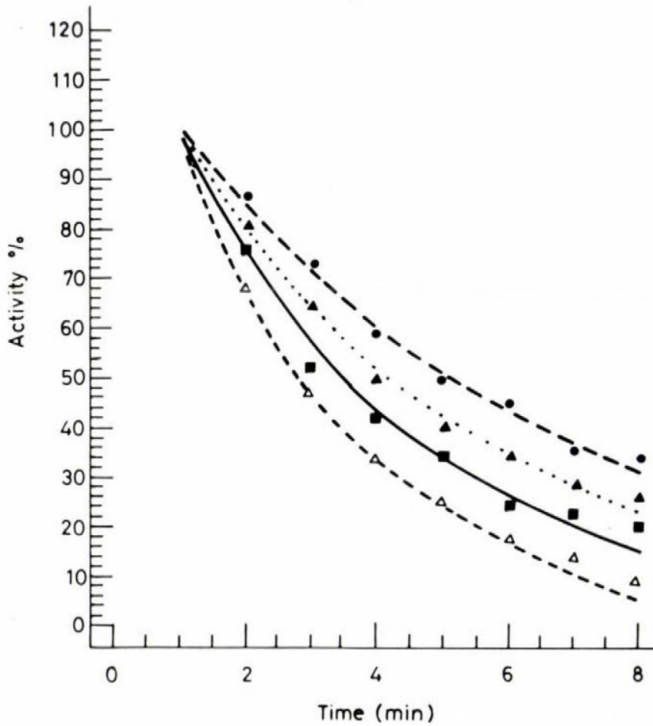


Fig. 4. The effect of X-ray on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. RES was studied 4 days after the radiation. Δ K_{control} : 0.2255 ± 0.0108 control $T_{1/2}$: 3.07 min; $\bullet$ $K_{9 \text{ Gy}}$: 0.1193 ± 0.0175 $T_{1/2}$: 5.81 min; $\blacktriangle$ $K_{8 \text{ Gy}}$: 0.1381 ± 0.0142 $T_{1/2}$: 5.02 min; $\blacksquare$ $K_{7 \text{ Gy}}$: 0.1818 ± 0.0121 $T_{1/2}$: 3.81 min

Results

Comparing the clearance curves of the first main group on Figs. 1 and 2, it can be seen that the whole dose of LPS and RD-LPS had a different effect. All 100 µg and 200 µg RD-LPS doses increased RES activity, but without a significant difference in effect. Whereas the low dose of LPS increased RES activity, the high dose decreased it. Endotoxin had a toxic effect in contrast to RD-LPS. There was a significant difference between these groups.

All three treatments (RD-LPS, trace elements and the combination of them) increased the granulopoietic activity of the RES. There are significant differences between the Granulopoietic Indices of the four groups (Fig. 3).

The effect of the different X-ray doses is shown in Fig. 4. All the 7, 8, 9 Gy X-ray radiation doses decreased RES activity. This effect is directly proportional to X-ray doses.

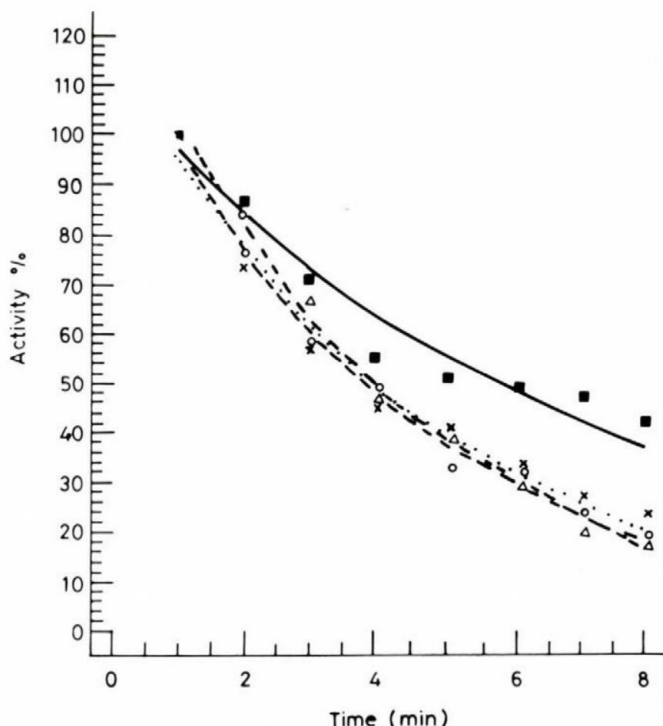


Fig. 5. The effect of X-ray with RD-LPS and trace elements treatment on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. ■ $K_8 \text{ Gy}$: 0.1381 ± 0.0142 $T_{1/2}$: 5.02 min; ○ $K_8 \text{ Gy} + \text{RD-LPS}$: 0.2422 ± 0.0104 $T_{1/2}$: 2.86 min; Δ $K_8 \text{ Gy} + \text{trace elements}$: 0.2461 ± 0.0108 $T_{1/2}$: 2.82 min; × $K_8 \text{ Gy} + \text{RD-LPS} + \text{trace elements}$: 0.2232 ± 0.0137 $T_{1/2}$: 3.11 min

We tried to moderate the RES-damaging effect of X-ray. Figure 5 shows that both RD-LPS and trace elements preparation had an advantageous effect. They could activate the RES functions in animals weakened by ethanol or X-ray.

Discussion

We developed a new method to investigate the granuloplectic activity of RES. This method causes less pain to the animals but it is very simple and allows the taking a lot of blood sample in contrast to other bloodsampling methods (from tail or sinus retroorbitalis).

We investigated some RES-injuring factors and managed to moderate these damaging effects by RD-LPS and trace element treatment.

The 8 Gy X-ray exerts an effect on the gastrointestinal tract [2] and, as a result, endotoxin enters the circulation. We modelled this effect in our first experiment.

Endotoxin has a membrane-damaging effect so that it decreases the RES activity [11]. The radiodetoxified endotoxin lacks this injurious effect, in fact, it can increase the RES functions [12]. Animals treated with X-ray became weakened, they lost water and had dyspepsia. In contrast to the results of Fred et al. [6] or Antonojevic [7], in our experiments X-ray irradiation impaired the phagocytosis. However, when rats were given the essential trace elements, they could synthesize a lot of important enzymes so their phagocytic cells could function. The effect of the RD-LPS has not exactly known yet but it can also activate phagocytosis. These animals can show more resistance to other infections. Based on our experiments it may assumed that the radiodetoxified endotoxin and trace elements can activate phagocyte cells of reticulo-endothelial system weakened by X-ray irradiation. It could be a possible therapy to protect patients suffering in radiation sickness from other bacterial infections.

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EFFECT OF RADIODETOXIFIED ENDOTOXIN AND TRACE ELEMENTS ON RETICULO-ENDOTHELIAL SYSTEM DAMAGED BY ETHANOL IN RATS

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For the demonstration of RES activity ^{99m}Tc labelled nano-albumin micro-colloid solution (prepared by OSSKI, Budapest, Hungary) was injected into the prepared jugular vein of narcotized rats and the clearance rate was investigated. The effects of various materials have been observed on RES in vivo. Continuous consumption of 20% ethanol solution for 15 weeks decreased the phagocytic activity. One hundred $\mu\text{g}/100\text{ g}$ body weight (i.v.) of the radio-detoxified endotoxin preparation (RD-LPS, TOLERIN) prepared from *Escherichia coli* endotoxin by ^{60}Co -gamma irradiation (150 KGy) increased the granulopoietic activity of RES of intoxicated animals. The preparation containing trace elements also increased the former activity and the treatment consisting of the use of both trace elements and RD-LPD was as effective as the RD-LPD per se.

Increased susceptibility to microbial infections as a result of acute or chronic ethanol consumption was observed in both human patients and experimental animals [1–4]. Green and Kass [5] demonstrated that aerosolized bacteria decreased the clearance from the lungs of intoxicated animals. Ethanol caused inhibition of PMN migration into the lung [6] and suppression of alveolar macrophage function by means of inhibiting their mobilization into the lung [7] and decreasing their adherence, phagocytosis and bactericidal activity [8, 9]. Experiments of Louria et al. [10] indicated that intoxication decreased the bacterial clearance by rat peritoneal macrophages. Incubation with ethanol in vitro inhibited the Fc-receptor function [11] and phagocytosis of human monocytes [12]. Livers of rats fed ethanol reduced clearance of perfused bacteria [13, 14]. Intoxicated rats had a depressed clearance of microaggregated albumin [15]. Serum albumin clearance was slower in alcoholics than in normal subjects [16].

In our previous study the effect of X-irradiation was investigated. Radiodetoxified endotoxin [17] and trace element pretreatment [18] improved the function of the reticulo-endothelial system damaged by X-radiation.

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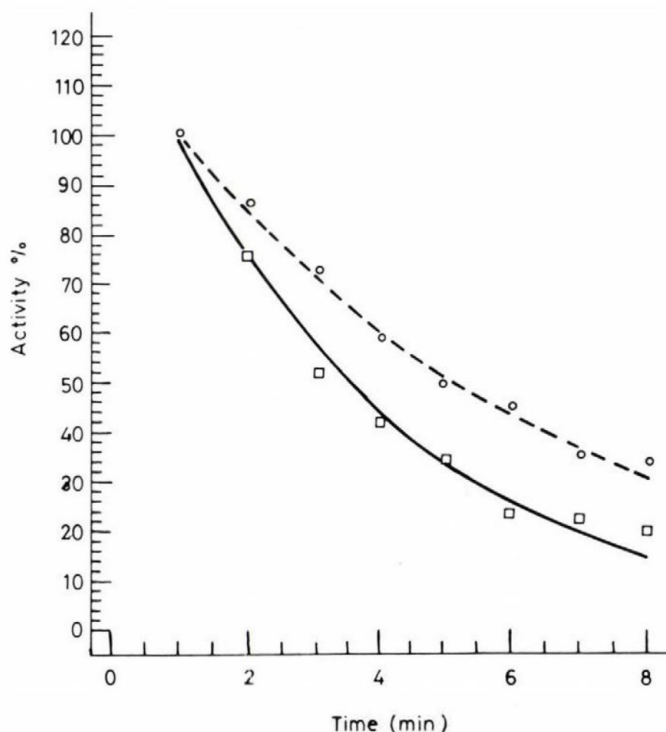


Fig. 1. The effect of ethanol consumption for 15 weeks on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. □ K_{control} : 0.2699 ± 0.0145 $T_{1/2}$: 2.57 min; ○ K_{ethanol} : 0.1701 ± 0.0052 $T_{1/2}$: 4.07 min

In the present study we observed the effect of ethanol intoxication on RES in vivo. We used the above-mentioned radiodetoxified-endotoxin (RD-LPS) and/or a special preparation containing trace elements to moderate these damaging effect of chronic ethanol consumption on RES system of the rats. There are few publications about combination these factors [19–21].

Materials and methods

Animals. We used 70 female Wistar rats from LATI (Gödöllő) weighting 140–150 g. They were fed with granulated chow (LATI, Gödöllő) and tap water ad libitum. Four groups of rats were given 20% ethanol instead of water during the experiment. Each group contained 10 animals. Animals used this study were maintained and used in accordance with the "Guide for the Care and Use of Laboratory Animals" (Hungarian Academy of Sciences).

Preparation of microaggregated nanoalbumin. The microaggregated ^{99m}Tc labelled nanoalbumin (NANOALBUMON) was prepared in "Frederic Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary. The particle size was 50–80 nm.

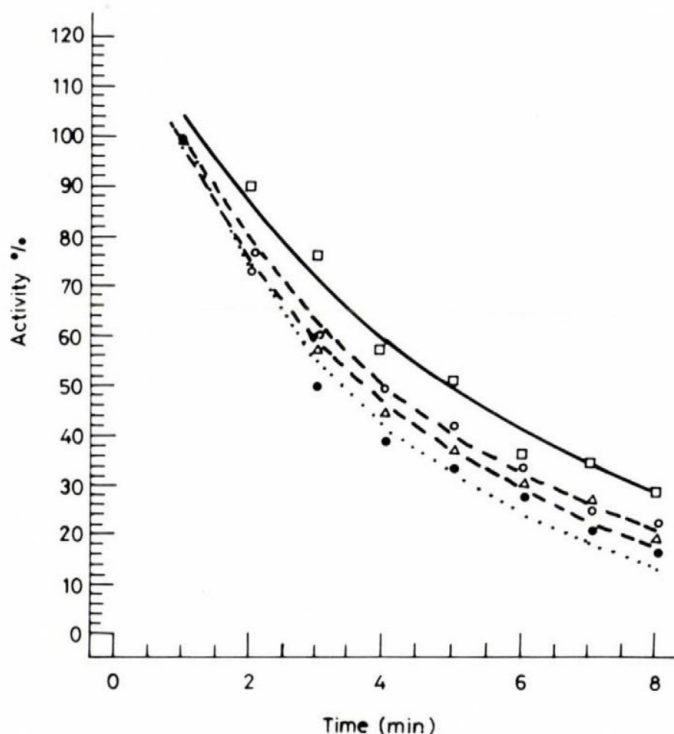


Fig. 2. The effect of RD-LPS and trace elements on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. $\square$ K_{control} : 0.1874 ± 0.0109 $T_{1/2}$: 3.70 min; $\circ$ $K_{\text{RD-LPS}}$ 100 µg: 0.2223 ± 0.0072 $T_{1/2}$: 3.12 min; Δ $K_{\text{trace elements}}$: 0.2312 ± 0.0098 $T_{1/2}$: 3.00 min; $\bullet$ $K_{\text{RD-LPS+trace elements}}$: 0.2670 ± 0.0159 $T_{1/2}$: 2.59 min

Preparation of endotoxin. The *E. coli* endotoxin (LPS) was prepared in our laboratory according to modified method of Westphal et al. [22].

Preparation of radiodetoxified endotoxin. The radiodetoxified endotoxin (RD-LPS, TOLERIN) was prepared in our laboratory from *E. coli* endotoxin irradiated with 150 kGy Co gamma-ray [23].

Ethanol consumption. Four groups of animals received 20% ethanol instead of water for 15 weeks.

Trace element treatment. A special trace element preparation (Béres Drops Plus, Béres Inc., Hungary) was given to 4 groups of animals through a probe at doses of 28 drops (2 ml) every day for 4 weeks. The trace element preparation contains Fe, Zn, Na, Mg, K, Cu, Mo, V, Ni, B, F, Cl, Co, glycerol, EDTA, L-(+)-tartaric acid, succinid acid, L-(+)-ascorbic acid.

Experimental groups. Group 1. Control. Group 2. Twenty per cent ethanol consumption for 15 weeks before the RES study. Group 3. 100 µg RD-LPS injected i.v. 24 h before the RES study. Group 4. Trace element treatment for 4 weeks before the experiment. Group 5. Combination of ethanol and trace element administration. Group 6. Combination of ethanol and RD-LPS administration. Group 7. Combination of ethanol, RD-LPS and trace element treatment.

Experimental method. Animals were anaesthetized by Nembutal (0.004 g/100 g body weight). Both v. jugularis were prepared, a canule (1 mm I.D.) was led into one of the veins. The 50 µg ^{99m}Tc labelled nano-albumin microcolloid was injected in the other vein. Eight blood samples were taken through the at intervals of 1 min.

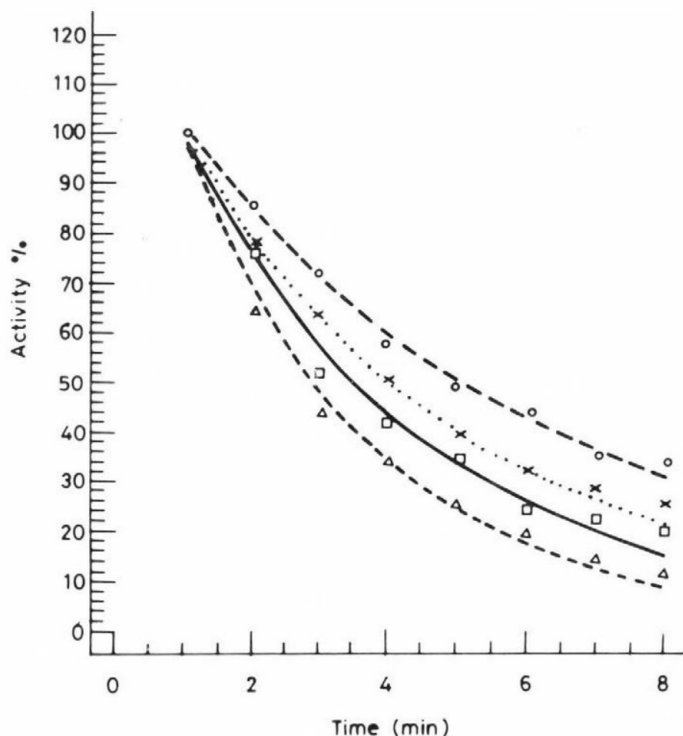


Fig. 3. The effect of ethanol with RD-LPS treatment on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. □ K_{control} : 0.2699 ± 0.0145 $T_{1/2}$: 2.57 min; Δ $K_{\text{RD-LPS 100 µg}}$: 0.3415 ± 0.0191 $T_{1/2}$: 2.03 min; ○ K_{ethanol} : 0.1701 ± 0.0052 $T_{1/2}$: 4.07 min; × $K_{\text{ethanol+RD-LPS}}$: 0.2206 ± 0.0127 $T_{1/2}$: 3.14 min

Calculation of results. The weight and radioactivity of the blood samples were measured and a calculation was made for 1 g of blood. Result for the first blood sample was regarded as 100%, the other samples were compared to this. Data were expressed in diagrams and exponential curves were fitted by STATGRAPHIC computer software. We compared the different Granulopectic Indices (K) [1]. The difference between this indices was subjected to a standard *t*-test ($p=0.05$)

Results

Long-term ethanol consumption had a damaging effect on phagocytic cells of the RES (Fig. 1). RD-LPS, continuous trace elements treatment and their combination activated the phagocytic capacity of the RES of healthy animals (Fig. 2). The effect of these preparations was compared on that of intoxicated animals. Both RD-LPS and trace elements activated phagocytosis of animals weakened by ethanol (Figs 3 and 4). Combination of these treatments had a stimulating effect on RES (Fig. 5). There was a significant difference between the clearance rate of the ^{99m}Tc labelled nano-albumin in the control groups and in the treated groups.

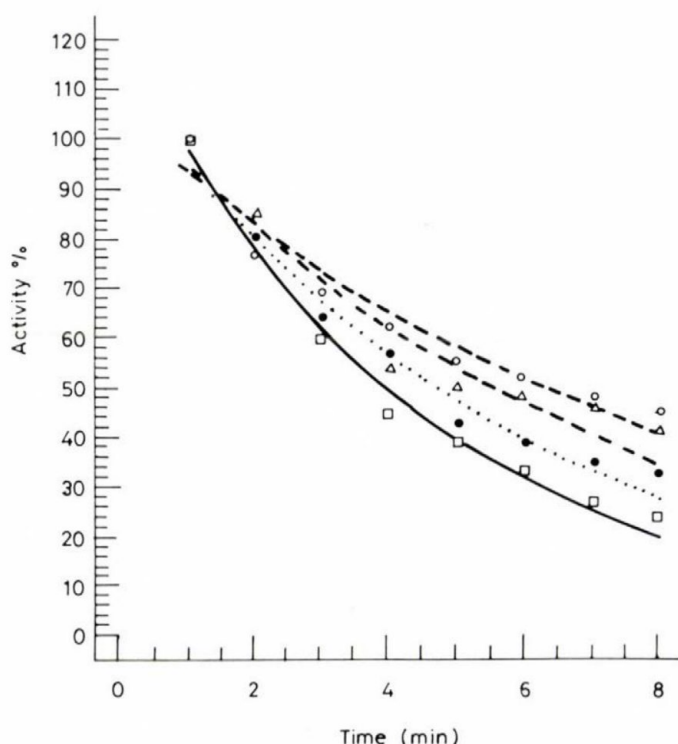


Fig. 4. The effect of ethanol with trace elements treatment on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. ● K_{control} : 0.2699 ± 0.0145 $T_{1/2}$: 2.57 min; ○ K_{ethanol} : 0.1701 ± 0.0052 $T_{1/2}$: 4.07 min; □ $K_{\text{trace elements}}$: 0.3269 ± 0.0054 $T_{1/2}$: 2.12 min; Δ $K_{\text{ethanol+trace elements}}$: 0.2107 ± 0.0078 $T_{1/2}$: 3.27 min

Intoxicated animals treated with RD-LPS or trace elements were not healthy but their phagocytic capacity was significantly larger than that in intoxicated animals without RD-LPS and/or trace elements. There were no significant differences between the effect of RD-LPS and trace elements or their combination.

Discussion

Long-term ethanol consumption has numerous damaging effects, for example alcoholic liver injury or increased susceptibility to infections. Kupffer cells phagocytose different kinds of particle in liver. The Kupffer cells are damaged by ethanol and, as a result, the RES activity decreases [9]. RD-LPS treatment activates these cells [17] and in this manner it can decrease the possibility of other infections. Trace element therapy, activating the whole RES system, might compensate for the lack of the function of Kupffer cells.

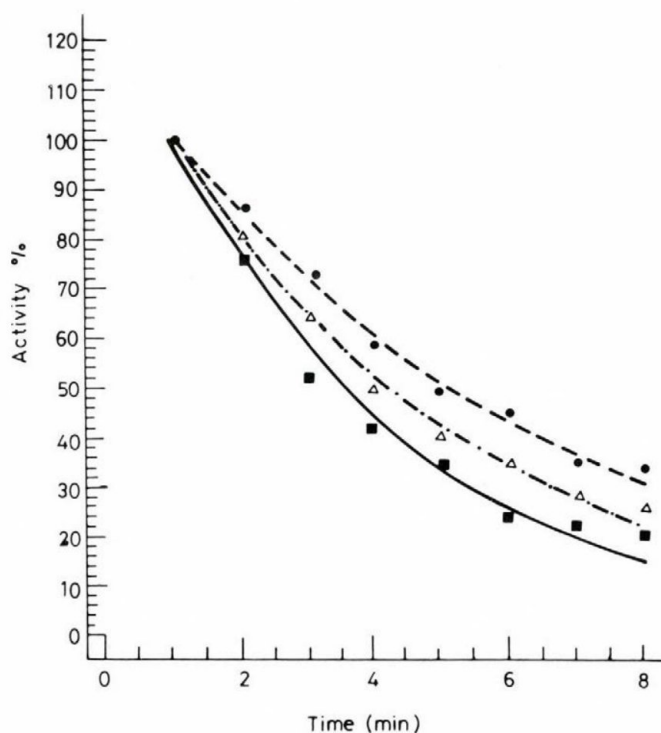


Fig. 5. The effect of ethanol with RD-LPS and trace elements treatment on clearance curve of 50 µg ^{99m}Tc labelled nano-albumin microcolloid. ■ K_{control} : 0.2699 ± 0.0145 $T_{1/2}$: 2.57 min; ● K_{ethanol} : 0.1701 ± 0.0052 $T_{1/2}$: 4.07 min; ▲ $K_{\text{ethanol+RD-LPS+trace elements}}$: 0.2016 ± 0.0034 $T_{1/2}$: 3.43 min

On the basis of these results, it would appear that the above-described treatments could activate reticulo-endothelial system, which is a part of the nonspecific resistance of the organism. Accordingly, RD-LPS and/or trace elements might be beneficial in protecting alcoholics from infections.

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PRODUCTION OF ANTIBODIES AGAINST ADENOVIRUS HEXONS IN *ESCHERICHIA COLI* BY RECOMBINANT PHAGES

(A NOTE)

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For the study of complex antigen structures, such as the adenovirus hexon capsomer, monoclonal antibodies proved to be very useful tools. Production and stabilization of monoclonal antibodies in a larger amount is complicated and requires special infrastructural background, if we apply the conventional hybridoma techniques. Recombinant DNA and gene amplification technology, however, has made it possible to clone antibody genes in bacteria, and to produce antibodies in bacterial cultures.

In our experiments a mouse hybridoma cell line was developed using crystallized adenovirus type 1 hexon, as immunizing antigen. The specificity of the antibody produced by the hybridoma against the adenovirus hexon was characterized previously [1, 2]. From the hybridoma cells mRNAs were isolated supposed to be transcribed mainly from the hexon specific immunoglobulin genes. The purification of mRNA was performed by oligo-dT-cellulose affinity chromatography. First-strand cDNA was generated from the mRNA by reverse transcription using random hexadeoxyribonucleotides. The synthesized first-strand antibody cDNA was used as a template for PCR amplification to generate suitable quantities for the antibody heavy and light chains encoding DNA for cloning. The amplification was carried out in two separated reactions with primer pairs which bind specifically to the conserved regions of the antibody genes. The amplified heavy (about 340 bp) and light (about 325 bp) chain DNA fragments were separately purified by agarose gel electrophoresis. After purification, the heavy and light chains were assembled into a single gene with a short DNA linker. This single antibody gene was then amplified with a set of oligonucleotide primers that introduced SfiI and NotI restriction sites to its 5 and 3 ends, respectively. Following amplification the antibody gene was purified and sequentially digested with the two enzymes to generate sticky ends for ligation into phagemid pCANTAB5 (Pharmacia) cloning vector. The phagemid also contains SfiI and NotI restriction sites and the gene too, coding for the phage M13g3p protein. The antibody gene was cloned

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into the vector adjacent to the g3 signal sequence for the expression as single chain antibody-g3p fusion protein. The cloned vector then was introduced into *Escherichia coli* JM109 bacterial cells by electroporation. Transformed *E. coli* cells were grown at 37 °C in a medium containing glucose and ampicillin. Only cells containing phagemid were grown. These cells were cloned by making isolated colonies and then the clones were infected with M13K07 helper phage to initiate phage replication and to yield recombinant phage. The phage contain phagemid pCANTAB5 vector with its specific adenovirus hexon antibody gene insert and displays the corresponding recombinant phage antibody as fusion proteins on its tip. Out of one hundred clones examined two have been shown displaying antibodies. The presence of antigen-reactive recombinant antibodies were detected and identified in ELISA, using 96-well microtiter plates coated with purified adenovirus type 1 hexons, and peroxidase-labelled sheep anti-M13 phage antibodies. The specificity of the phage-antibodies proved to be identical with that of the original hybridoma antibodies. In the antibody positive phage the presence of the antibody-gene was proven by digestion with NotI enzyme.

This system of production and characterization of single chain antibodies may give new perspectives in molecular immunology and virology as well as in other fields of biomedical sciences.

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